The Evolutionary Ecology of Mutualism

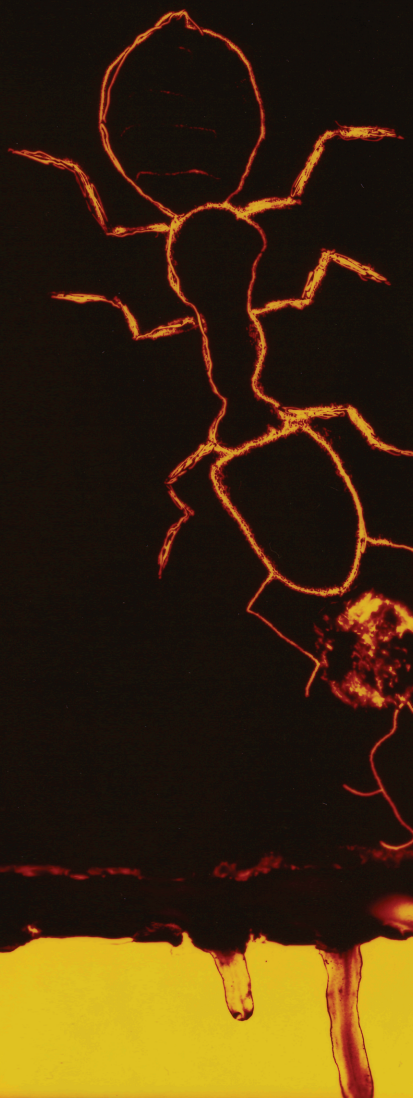
Ivens, A.B.F.

2012

document version
Publisher's PDF, also known as Version of record

The evolutionary ecology of mutualism

Aniek B.F. Ivens



Stellingen behorende bij het proefschrift

The evolutionary ecology of mutualism

door

Aniek Barbara Françoise Ivens

1. Three common species of root aphids farmed by the ant *Lasius flavus* are characterised by clonal reproduction, rare dispersal and (genetic) monocultures at the lowest spatial scale. Such a combination of symbiont characteristics is commonly found in farming mutualisms and likely constitutes a general set of necessary conditions for the evolutionary stability of these systems.
   This thesis, Chapters 1, 2, 3, 4 and 8; *e.g.* Poulsen & Boomsma 2005 *Science*; Aanen *et al.* 2009 *Science*

2. 'Cattle farming' by the ant *L. flavus* can be considered 'niche construction', since the 'in-house' availability of cattle-provided sugars and proteins allows for an exceptionally high density of this species in salt-marsh habitats; this domestication process can thus be considered analogous to the dramatic increase in human population densities after our ancestor's cultural transition to a farming lifestyle.
   This thesis, Chapter 4; Larsen 1995 *Ann. Rev. Antropology*; Laland & Boogert 2010 *Ecol. Economics*

3. Despite the importance of partner choice for the stability of many mutualisms, the low aphid diversity observed in *L. flavus* mounds can more parsimoniously be attributed to passive mechanisms of dispersal and local asexual reproduction in the root aphids rather than to active choice by the ants.
   This thesis, Chapters 2, 4, 7 and Box A

4. Choosing to stick with cooperative partners does not automatically lead to the evolution of more cooperation.
   This thesis, Chapters 6 and 7

5. Models involving conditional strategies can yield counter-intuitive results and novel 'out-of-the-box' insights, which can give rise to future research avenues of explicit hypothesis testing.
   This thesis, Chapters 6 and 7

6. Low cost-benefit ratios and asymmetry in cooperative investments are key ingredients for successful mutualism.
   This thesis, Chapters 7, 8

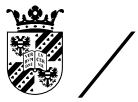
7. Up to 9.5 million tons of food is wasted in The Netherlands annually; this is largely because little value is given to food throughout the complete food chain. Therefore, an important lesson remains to be learned from yellow meadow ants: "*De* [*L. flavus*] *mieren kennen er* [*het vee*] *de waarde van, het is hun schat*" (In: "De wijsheid der mieren" by Prof. F.J.J. Buytendijk, 1922).
   De Nationale DenkTank 2012

8. Parallel study of multiple study systems is needed to identify general patterns in biology. However, conducting a classical, descriptive study can be a handicap to (young) scientists developing novel study systems in evolutionary ecology, since the highest ranking journals prefer studies involving fancy new research technology. Yet, basic descriptive studies of a system's biology are indispensable for making the application of these techniques to novel study systems worthwhile for between-system comparison.

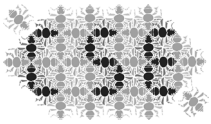
9. PhD curricula could be better adapted to the future careers of the students following them, because they appear mostly designed to prepare students for a career in science, while >30% of the students pursue a career outside academia directly after completion of their PhD.
   Sonneveld *et al.* 2010, IVLOS, Larsen & Lubbe 2008, VSNU

10. People who say 'yes' are rewarded by the adventures they have; people who say 'no' are rewarded by the safety they attain.
    "Impro" by K. Johnstone, 1979

The evolutionary ecology of mutualism



rijksuniversiteit
groningen



This PhD project was carried out at the Theoretical Biology Group, which is part of the Centre for Ecological and Evolutionary Studies of the University of Groningen (The Netherlands), according to the requirements of the Graduate School of Science (Faculty of Mathematics and Natural Sciences, University of Groningen). Part of this project was also carried out at the Centre for Social Evolution, Department of Biology, University of Copenhagen, Denmark.

Aniek Ivens received a PhD-grant from the Faculty of Mathematics and Natural Sciences following top-master programme Evolutionary Biology. The research was supported by the Pieter Langerhuizen Stipedium and by Stichting Nicolaas Mulerius Fonds. The printing of this thesis was partly funded by the University of Groningen and the Faculty of Mathematics and Natural Sciences, University of Groningen.

Lay-out and figures:	Dick Visser
Cover art work:	Marlies Hulzebos
Cover design:	Aniek Ivens
Pictures:	All by Aniek Ivens, except for Figure 1.3C by Thijs Janzen and the picture on page 192 by Jeroen Kuijpers
Printed by:	Ipskamp Drukkers, Enschede

ISBN: 978-90-367-5828-4
ISBN: 978-90-367-5827-7 (electronic version)

RIJKSUNIVERSITEIT GRONINGEN

The evolutionary ecology of mutualism

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 23 november 2012
om 16.15 uur

door

Aniek Barbara Françoise Ivens

geboren op 22 augustus 1983
te Heerlen

Promotores:	Prof. dr. F.J. Weissing
	Prof. dr. J.J. Boomsma
	Prof. dr. I.R. Pen
Beoordelingscommissie:	Prof. dr. N.E. Pierce
	Prof. dr. K.R. Foster
	Prof. dr. B.J. Zwaan

De mier
Ik heb sinds kort een mier,
ik noem het beessie Bartje,
't is zo'n mieren-mini-dier,
hij heeft een héél klein hartje,
maar dikwijls denk ik, sapperloot,
wat leeft er in die kleine?
't Geheim in hem is nét zo groot
of groter dan het mijne.

- Toon Hermans (1983)-

Voor Barbara en François

Contents

CHAPTER 1

General introduction and thesis outline

Aniek B.F. Ivens

MUTUALISM

Mutualism, an interaction between different species that is beneficial for all actors, is widespread throughout nature. To a large extent, mutualism has shaped, and is still shaping, life on this planet. In fact, life as we know it would not have existed without mutualistic relationships. For example, all eukaryotic life is based on ancient endosymbiotic mutualisms between its cells and formerly independent microorganisms (*e.g.* mitochondria, plasmids) (Margulis 1971). Other mutualisms are known to have major impact on ecosystem stability, such as specialized interactions between flowering plants and their pollinators, or seed dispersal by birds, mammals and other animals. The mutualistic relationship between humans and their agricultural crops and domesticated animals was key to the dominant role our species is now playing on our planet (Larsen 1995; Stachowicz 2001; Leigh 2010).

Already from these few examples it becomes clear that mutualism comes in many different types and forms. Known mutualisms range from one-to-one interactions [*e.g.* cleaner fish that clean their client fish (Bshary & Grutter 2002; Bshary *et al.* 2002; Bshary & Grutter 2006)] to mutualistic networks with many partners interacting with many, such as several species of pollinators interacting simultaneously with several species of flowering plants (Bascompte *et al.* 2006). Some mutualisms are associations for life or even multiple generations [*e.g.* endosymbionts such as our gut-bacteria (Bäckhed *et al.* 2005; Booijink *et al.* 2007) or fungus-growing ants that farm their mutualistic fungus in their nest (Mueller 2002; Poulsen & Boomsma 2005)], whereas other interactions are only short-termed [*e.g.* fig-wasps that only complete part of their life cycle inside fig fruits (West & Herre 1994; Jander & Herre 2010)]. In addition, mutualisms can also differ in how dependent partners are on the interaction for survival and maximization of their reproductive success. In obligate mutualisms partners fully depend on each other and the interaction, whereas in facultative mutualisms the partners' fitness is enhanced by the interaction, but partners can also survive and reproduce on their own. Lastly, the nature of goods or services exchanged differs significantly between mutualisms: some rely on transportation, others on nutrition or protection (Bronstein 2001).

Despite its importance for all aspects of life and its huge diversity, mutualism has historically received much less attention than other ecologically and evolutionarily important processes, such as competition, host-parasite interactions, trophic relationships, or within-species cooperation. Only in the last two decades, ecologists and evolutionary biologists are starting to realize that the eco-evolutionary causes, consequences and stability of mutualism are still poorly understood (as pointed out by Herre *et al.* 1999). With this dissertation, I aim to contribute to a better general understanding of the evolutionary ecology of mutualism. To this end, I have combined two complementary approaches: a specialized *empirical approach* via a detailed case study of a farming mutualism, that of the underground ant *Lasius flavus* tending various species of root aphids inside its nest (Chapters 2-5), and a more general *theoretical approach*, via the development of models about the evolutionary emergence and stability of between- and within-species cooperation (Chapters 6, 7).

This chapter serves as an introduction to both approaches. First, I will introduce the evolutionary problem of cooperation, with emphasis on mutualism, by briefly reviewing

the existing body of literature and highlighting the most important advances so far. I will also introduce the most relevant terms (Table 1.1). This is followed by an introduction to the particular group of farming mutualisms that my empirical studies address. Lastly, I will give a brief overview of the aims and contents of the thesis.

THE PUZZLE OF WITHIN-SPECIES COOPERATION

Natural selection acts through competition for reproduction and survival. How then can individuals evolve to help others with costly behaviour, whereas it would be more beneficial to reap the benefits without paying the costs? Cooperation poses a particular problem if social dilemmas arise, *i.e.* when the group as a whole would profit most if all members would contribute to a common good, but where individual members would profit most by not contributing, that is by 'cheating'. But even in situations where all participants have an individual incentive to cooperate, the evolution of cooperation is not self-evident, for example when the cost and benefits of the interaction are asymmetrically distributed. The widespread occurrence of cooperation and its evolution pose a problem that has been one of the most hotly debated issues in evolutionary biology for several decades now (among others Hamilton 1964a; 1964b; Trivers 1971; West-Eberhard 1975; Axelrod & Hamilton 1981; Brown 1983; Queller 1985; Bull & Rice 1991; Fletcher & Doebeli 2009, recently reviewed in Sachs *et al.* 2004; West *et al.* 2007; Bourke 2011). The controversy evoked by a recent paper on the evolution of eusociality (Nowak *et al.* 2010; Abbot *et al.* 2011; Boomsma *et al.* 2011; Ferrière & Michod 2011; Nowak *et al.* 2011) indicates that even today this problem remains a hot topic. Over the years, major conceptual advancements have been made to resolve this evolutionary puzzle. Quite a number of reviews appeared that tackled classification of the mechanisms that have been recognized to promote the evolution of cooperation (beneficial interactions between both actors) and, more specifically, altruism (within-species cooperative behaviour that is costly to the partner and beneficial to the recipient) (Sachs *et al.* 2004; Nowak 2006; West *et al.* 2007). In my view these mechanisms can be crudely divided in two groups: the first consisting of mechanisms that promote *non-random associations* of interacting partners and the second consisting of mechanisms for the expression of *conditional strategies*. I will now discuss these two groups in more detail separately.

Mechanisms promoting non-random associations

As opposed to when interactions take place between random partners, interactions in non-random associations take place between partners with a similar type of strategy, *e.g.* cooperative individuals interact with other cooperative individuals and non-cooperative ones with other non-cooperative individuals. Mechanisms that promote such positive assortment of cooperators include *kin selection*, which is relevant for the evolution of altruism (Hamilton 1964a; 1964b) and passive assortment mechanisms (Queller 1992; 1994; Fletcher & Doebeli 2009) such as population viscosity, that cause cooperative individuals to encounter other co-operators more often than cheaters and vice versa (as seen for example in bacterial biofilms (Hallatschek *et al.* 2007; Nadell *et al.* 2010). Positive assort-

ment of cooperators reduces the benefits to be gained by cheaters: because cheaters and cooperators mix to a lesser extent, cheating individuals cannot exploit the cooperative community.

Conditional strategies

The second group of mechanisms consists of *conditional strategies*, in which the behaviour of an individual depends on its social context: what others do and who others are. A classical example of cooperation through conditional strategies is *reciprocity*, where individuals have the opportunity to leave interactions when cheated upon (Trivers 1971). Well-known examples of the study of cooperation through reciprocity are the numerous studies dealing with the *Iterated Prisoner's Dilemma (IPD)* in game theory. The non-iterated version of this game is the prototype example of a social dilemma involving two players (the 'prisoners'), for whom, although cooperation ('not confessing a common crime') would the best option for both players, defecting ('confessing') remains the dominant strategy, because it yields individually the highest benefit if one does not know what the other participant will do. As a consequence, the game-theoretical solution ('both players confess') corresponds to an outcome where both players are worse off than if they both had cooperated in denial. It has been shown that the dilemma can – at least partly – be resolved if the players do not play the game once but repeatedly (potentially infinitely). Some of the solutions correspond to a cooperative outcome. Much discussed strategies leading to cooperation are 'Tit-For-Tat' and related strategies (*e.g.* Axelrod & Hamilton 1981; Selten & Hammerstein 1984; Nowak & Sigmund 1993), where each player decides whether to cooperate or defect based on previous experiences with the partner. The chance to decide to continue an interaction or abandon a defecting partner has been termed *partner fidelity* in mutualism literature (see below). Another well-known example of conditional strategies promoting cooperation is the green beard theory (among others Hamilton 1964b; Dawkins 1976; Jansen & van Baalen 2006), in which individuals only cooperate with others that carry a trustworthy 'green beard' marker that is correlated with cooperativeness. Besides theoretical studies, genes that operate together with a kin effect as 'green beard' – genes have now also been empirically identified, for example in social amoeba *Dictyostelium* and red fire ants (Keller & Ross 1998; Queller *et al.* 2003). A last major category of conditional strategy–mechanisms is part of the *Biological Market Theory (BMT)* (Noë & Hammerstein 1994). BMT applies market mechanisms known from economy to biological contexts and is based on the idea that individuals do not engage in interactions with everybody, but only do so with cooperators (*partner choice*). Partner choice, partner fidelity and their possible interaction have, however, seldom been studied simultaneously. In my thesis, I will address the relative importance of partner fidelity and partner choice in joint evolution with intraspecific cooperation (Chapter 6).

Both groups of mechanisms listed can lead to the same end results: positive assortment of co-operators. Therefore, these mechanisms are not mutually exclusive. Over the last few years, attention of the literature shifted from studying altruism towards cooperation between non-kin and focussing on conditional strategies. Nevertheless, kin selection theory still provides one of the most important explanations for the evolution of cooperation in nature (with the potential exclusion of humans) (Hamilton 1964a; 1964b; Bourke 2011).

THE EVEN GREATER PUZZLE OF BETWEEN-SPECIES COOPERATION

Kin selection theory is based on interacting partners carrying with a high probability identical genes (through *identity by descent (IBD)*). However, in interspecific cooperation IBD cannot apply because interacting individuals of different species each have separate gene pools. The existence of a wide diversity of mutualisms in which different species seem to help each other thus poses several unsolved evolutionary problems. In fact, Darwin (1859) already pointed out in his chapter on difficulties on his theory, that no species will ever evolve traits or behaviour for the exclusive good of another species:

> *'Natural selection will produce nothing in one species for the exclusive good or injury of another; though it may as well produce parts, organs, and excrements highly useful or even indispensable (...) to another species, but in all cases at the same time useful to the owner.'*

Both the *evolutionary emergence* of mutualism and the *maintenance* of mutualism remain poorly understood. How does cooperation between individuals of different species come off the ground to start with? Then, once a mutualistic interaction has been established, natural selection will continue to act on the separate partners and seldom on the mutualism as a whole. The partners may thus both be selected to reap the benefits from the interaction without paying the costs of investment, causing cheating behaviour and conflicts of interests between the partners (Herre *et al.* 1999; Bronstein 2001; Bergstorm *et al.* 2002; Sachs & Simms 2006). For this reason, mutualism has previously also been defined as *'mutual exploitation with net benefits for both'* (Herre *et al.* 1999). How then can mutualism emerge? And what prevents subsequent mutualism breakdown when faced with cheaters and conflict between partners? Several mechanisms have been put forward that can promote mutualism emergence and maintenance. These mechanisms are not mutually exclusive and can be simultaneously at work in shaping interspecific interactions. Here, I will give a brief overview, albeit not exhaustive, of the main theoretical concepts concerning mutualism evolution and stability.

By-products and the importance of cost-benefit ratios

Many mutualisms may have started off as a *by-product mutualism*: a selfish act (*i.e.* performed solely for one's own good), which results in a good or a service (the 'by-product') that is incidentally beneficial for a partner species (West-Eberhard 1975; Brown 1983; Connor 1995; Leimar & Connor 2003). Typically, these interactions start as asymmetric interactions: one partner produces a by-product from which the other partner profits (such an interaction is also called *commensalism*). Through co-adaptation of both partners this exchange of by-products can then further evolve into mutualism (Connor 1995). In fact, some have argued that maybe all mutualisms that are known today started with by-products in at least one of the partners (Connor 1995). Well-known examples of by-product mutualism are ant-homopteran interactions in which homopterans (aphids, coccids) provide their faeces (honeydew) to ants, which provide protective services in return (Connor 1995; Leimar & Connor 2003; Leigh 2010). For the by-product producing species,

the *cost-benefit ratio* of the interaction is very low: it does not incur (almost) any costs producing the good, but does receive the benefits of the goods or services gained in return. A low cost-benefit ratio of the interaction might thus be a good incentive for a species to engage in mutualism. Also, a low cost-benefit ratio makes the interaction less prone to cheating as there are no large costs to be avoided in the first place. Indeed, in theoretical models, cost-benefit ratios have been shown to play a major role in the evolution of mutualism in several studies (Foster & Wenseleers 2006; Lehmann & Keller 2006). In fact, many regard mutualism and parasitism as two ends of a single continuum as is also illustrated by Herre *et al.*'s (1999) definition of mutualism as mutual exploitation (Bronstein 1994; Hoeksema & Bruna 2000; Oliver *et al.* 2009). Where exactly the interaction is placed on

Table 1.1 Glossary of used terms.

Term	Definition
Altruism	Within-species interaction that is beneficial to the recipient and costly to the actor
Parasitism	Interaction between different species that is beneficial to one partner and costly to the other
Mutualism	Interaction between different species that is beneficial for all (both) partners
Commensalism (by-product mutualism)	Interaction between different species that is beneficial to one partner and has a neutral effect on the other partner
Farming mutualism	Mutualism in which one partner promotes the growth of the other partner on which it relies for food
Symbiosis	Interaction in which different species live closely together, often lifelong. Symbiosis can be parasitic or mutualistic
Host/endosymbiont mutualism	Asymmetric interaction between (often) a large, long lived species (the host) and a small, short lived species (the symbiont), that either lives inside the body of the host or in a structure built by the host
Obligate/facultative mutualism	In obligate mutualisms, both partners are dependent on the interaction for survival and/or reproduction. In facultative mutualism, partners are not strictly dependent on the interaction and on each other.
Horizontal transmission	Transmission of symbionts between different hosts (can happen within a single host generation)
Vertical transmission	Transmission of symbionts from a host to its descendants (often during host reproduction)
Relatedness	Genetic correlation between interacting individuals of the same species
Between-species genetic correlation	Correlated occurrence of genes in different species
Partner fidelity	The possibility for an interacting individual to abandon or continue the current interaction dependent on the partner's past behaviour
Partner choice	A mechanism that allows individuals to differently interact with cooperative and non-cooperative partners

the continuum depends on its cost-benefit ratio. Over evolutionary time partners may move along this continuum and the nature of the interactions can change as the partners co-evolve. The continuum can be best thought of as a balance that can tip to both sides. Parasitism can evolve from mutualism if two partners are mutually dependent and thus 'trapped' in the interaction and one of the partners evolves to exploit the other (Sachs & Simms 2006). Alternatively, parasitism can also be a starting point for mutualism if the parasitized host manages to evolve to profit from the presence of the parasite (Yamamura 1993; Aanen & Hoekstra 2007).

Generalized kin selection

Genetic *relatedness* (through IBD) is not possible between individuals of different species. However, genetic correlation across species boundaries (= *between-species genetic correlation*) can occur when two species enhance each other's productivity because individuals of both species with similar traits tend to co-occur and interact. For example, in case of traits involved in cooperation, this happens when between-species assortment causes cooperative individuals of one partner species to tend to interact with cooperative individuals of the other species (Queller 1985; Frank 1994; Doebeli & Knowlton 1998; Fletcher & Doebeli 2009). The presence of this between-species genetic correlation can thus promote interspecific cooperation.

Reciprocity

Reciprocity, as discussed in the previous section on within-species cooperation, has been put forward by Trivers (1971) (under the misleading name of 'reciprocal altruism') as a mechanism that could favour cooperation between unrelated individuals, also of different species. In the vast body of work inspired by this idea, the framework of the IPD has also been applied to the study of mutualism in several theoretical models, even though IPD assumes a symmetric interaction whereas most mutualisms are asymmetric (among others Bull & Rice 1991; Doebeli & Knowlton 1998; Hoeksema & Bruna 2000). Nevertheless, for this framework of repeated interactions to apply in nature, at least one or both of the following requirements needs to be fulfilled: (1) the partners have the ability to alter their behaviour in response to the outcome of previous interactions, *i.e.* break up interactions if desired and (2) to choose with whom to interact. These two requirements each correspond to one of the factors put forward by Bull & Rice (1991): (1) *partner fidelity* and (2) *partner choice*. These factors have come to play a large role in theoretical as well as empirical approaches to mutualism and are still debated to date (Weyl *et al.* 2010; Kiers *et al.* 2011; Weyl *et al.* 2011). I will discuss these factors in detail in the next paragraphs.

Partner fidelity

Partner fidelity translates to whether a partner will still be available for interaction in the next round; in other words, partner fidelity offers interacting partners the possibility to choose whether to continue or abandon an interaction. If partners repeatedly choose to continue the interaction, a *partner fidelity feedback* can occur. This long-term series creates a positive feedback loop between the fitness of the two partners: cooperation will indirectly, via its partner, benefit the actor (Bull & Rice 1991; Foster & Wenseleers 2006; Weyl

et al. 2010). Several mechanisms can favour the establishment of partner fidelity feedback in nature, all of them causing the interacting partners to be connected in space and/or time. For example, partners can co-disperse (in space) or co-reproduce (in time). If cooperative individuals of partner species tend to be spatially or temporally clustered, both species will indirectly gain benefits from their own investment into cooperation with the other species, because these benefits are likely to be returned, hence partner fidelity feedback can take effect.

Partner choice and sanctioning

Partner choice allows actors to discriminate between individuals of the partner species and specifically direct cooperative actions towards cooperative individuals of the partners (Bull & Rice 1991). Partner choice thus can take two forms: selection of partners before engaging in interaction [so called 'screening', known from bobtail squids which selectively allow fluorescent bacteria to enter their body, (Archetti *et al.* 2011)] and sanctioning of non-cooperative partners [known from client fish which reject nibbling cleaner fish (Bshary & Grutter 2002) and plants which cut off nutrient supplies to bacteria that do not fix enough nitrogen for them (Kiers *et al.* 2003)]. Partner choice and sanctioning have been extensively studied, both theoretically [mainly as part of the BMT (Noë & Hammerstein 1994)] and empirically [in among others cleaner fish, plant – rhizosphere systems and the bobtail squid (Bshary *et al.* 2002; Kiers & Denison 2008; McFall-Ngai 2008)].

In this thesis, I modelled the joint evolution of partner fidelity, partner choice and mutualism (Chapter 7). The evolution of within – and between-species cooperation have historically been studied separately from each other, although similar if not identical mechanisms may be at play. By deriving very similar models for within-species cooperation (Chapter 6) and between-species cooperation (Chapter 7), I will be able to closely compare the results and we can gain further insight in the similarities and the differences between these two processes.

FARMING MUTUALISM

Maybe it is simply not possible to cover the full diversity of mutualisms in a single theoretical framework. It is certainly not possible to cover the huge diversity of mutualisms in a single thesis. In the empirical part of this thesis, I therefore consider a particular kind of mutualism, farming mutualism. In this section, I will zoom in on these interactions more closely.

In farming mutualisms, the host partner promotes the growth of a symbiont that it depends on for food. This dependence includes both mutualistic systems in which the host feeds on goods produced by the symbiont and systems in which the host consumes the symbiont [the latter is commonly called 'cultivation mutualism' (Hata & Kato 2006)]. Many examples can be found in nature and these systems often show interesting parallels to human practices of cattle breeding and crop growing. For example, several insects are known to actively grow fungus as a crop in their nest, such as the leaf-cutter ants (Weber 1972; Mueller *et al.* 2005; Poulsen & Boomsma 2005), fungus-growing termites (Aanen

et al. 2002; Aanen *et al.* 2009) and ambrosia beetles (Farrell *et al.* 2001; Biedermann & Taborsky 2011). Other examples of crop growing include damselfish keeping gardens of multiple species of algae, which they actively protect from other grazers (Hata & Kato 2006; Hata *et al.* 2010) and even *Dictyostelium* amoeba that prudently farm the bacteria they eat (Brock *et al.* 2011). The best known examples of 'cattle breeding' are many different species of ants tending above ground or below ground homopterans for 'milk' (honeydew) and sometimes 'meat' (Way 1963; Stadler & Dixon 2005; Stadler & Dixon 2008; Ben-Dov & Fischer 2010). These systems differ in how dependent participants are on the interaction; both hosts and symbionts can be found that are obligately dependent on the other, but farming mutualisms can also be of facultative nature.

Farming mutualisms are inherently asymmetric: often the host species is larger, longer lived and engages in the interaction as a single individual (or colony). The symbiont species interacts often as a group of multiple individuals, has a short generation time and is smaller. Moreover, especially in systems where the host consumes the symbiont, the benefits to be gained by the host from the interaction stem directly from the ultimate sacrifice of the symbiont: giving its life. Generally, where species rely so closely on each other for survival and reproduction in an asymmetric interaction, the interests of the partners will never be fully aligned and conflict lures around every corner. Because it is often the host that is in control over the symbiont, the three main arenas of host/symbiont conflict in farming mutualisms concern the symbiont life history characteristics (1) symbiont reproductive mode, (2) symbiont transmission and dispersal, and (3) symbiont diversity (Frank 1996; Herre *et al.* 1999; Mueller 2002). Indeed, host control over mutualistic symbionts is thought to be essential for evolutionary stability of mutualisms (Frank 1996; Herre *et al.* 1999; Sachs *et al.* 2004; Archetti *et al.* 2011). However, are the hosts really in complete control and what outcomes of these conflicts are preferred by host and symbiont? Below, I will introduce these three conflicts, their possible outcomes and host/symbiont perspectives in general (Table 1.2). After that, I will discuss how they potentially apply to my study system of ants farming root aphids, after having introduced that system in detail.

Symbiont reproduction

In terms of reproductive mode, it would be in the interest of the host to prevent the symbiont from sexually reproducing: energy allocation to sexual reproduction is avoided and beneficial combinations of genes are preserved, because they are not reshuffled by recombination. For the symbiont it would be beneficial to sexually reproduce to avoid inbreeding effects and remain resilient in changing environments (Table 1.2). However, when hosts provide very stable niches by creating a *protective environment* to symbionts (Law & Lewis 1983; Law 1985; Wulff 1985), sexual reproduction might no longer be the preferred option for symbionts the environment no longer changes and the benefits of asexual reproduction (higher reproductive rate, not having to search for a mate) can take precedence. In this case, host control may thus emerge as a by-product of genotypic predictability. Indeed, several symbiotic bacteria such as *Buchnera* are both under host control through asexual, vertical transmission and enjoy highly predictable growth conditions in specific host tissues (Moran *et al.* 2008). Fungus-growing termites are known to suppress sexual reproduction of their fungus except for short periods each year, and fungus-growing ants normally sup-

press sexual reproduction of their fungus permanently, so that clonal vertical transmission is the rule (Weber 1972; Mueller *et al.* 1998; Bot *et al.* 2001; Aanen *et al.* 2009, but see Mikheyev *et al.* 2006).

Symbiont transmission and dispersal

Symbionts can be either *horizontally* or *vertically* transmitted. Under horizontal transmission, symbionts disperse independently from their host. Typically, at the beginning of a host generation, hosts associate with symbionts that are unrelated to symbionts with which their parents were associated. Under vertical transmission, symbionts are transmitted to descendants of their previous hosts during host reproduction. Vertical transmission is in the interest of both host and symbiont on the long-term, because it allows co-evolution between the mutualistic partners and hence productivity of the system can increase. Horizontal transmission, however, allows the symbiont on the short term to escape less beneficial interactions and to explore new habitats and avoid competition with close relatives (Hamilton & May 1977). Also, horizontal transmission allows the host to choose among potential symbionts and select the best partners (Table 1.2). Vertical transmission occurs in many mutualisms, ranging from endosymbionts such as *Buchnera* referred to above to ectosymbionts such as the fungus-growing attine ants in which virgin queens carry a piece of fungus from their natal nest on their nuptial flight (Weber 1972, but see Poulsen *et al.* 2009), two species of fungus-growing termites which carry asexual fungus spores on their mating flight (Korb & Aanen 2003) or ant-tended coccids that climb the back of virgin queens ready for take-off (Hölldobler & Wilson 1990). There are also plenty of examples of horizontal symbiont transmission, such as the fungus of several fungus-growing termites (Aanen *et al.* 2002) and above-ground aphids tended by ants (Yao 2010; Vantaux *et al.* 2011a).

Symbiont diversity

The conflict on symbiont diversity deals with the question of *polyculture* versus *monoculture*, a topic that is also debated with respect to human agriculture. From the symbiont's viewpoint, some diversity might be preferred, because this would prevent competition with close relatives and the chance to outcompete non-relatives (Frank 1996). Mutualistic hosts may, however, favour predominant or exclusive monocultures if coexistence of multiple strains or species within the same host allows costly contest competition (Frank 1996) or free-riding by underperforming symbionts, leading to a direct reduction in overall productivity (*e.g.* Bronstein 2001; Kiers & Denison 2008). Moreover, competition among symbionts may also select for virulent competitive traits that will indirectly also harm the host (Wulff 1985; Frank 1996). In addition, the high symbiont relatedness encountered in monocultures will allow the host to effectively select productive symbionts through artificial selection. Also, uniform conditions allowed by monoculture may increase mutualism productivity in undisturbed environments. This latter argument, however, remains debated, because a community of multiple symbionts might also offer a broader spectrum of services or might be less vulnerable to parasites (*e.g.* van Borm *et al.* 2002). Host control over symbiont diversity generally happens through specific mechanisms of symbiont screening upon admission (Archetti *et al.* 2011) or symbiont rewarding/sanctioning in proportion to

Table 1.2 Arenas of conflict in farming mutualisms, possible outcomes and benefits from host and symbiont perspective.

Arena of conflict	Possible outcomes	Host perspective	Symbiont perspective
Symbiont reproduction	Asexual	+ Beneficial gene combinations are preserved (beneficial in stable environments) + Less productivity loss due to symbiont energy allocation into sexual reproduction	+ All genes are passed on to the next generation + No energy and time loss due to searching for mates
	Sexual	+ Symbionts with higher adaptive potential (beneficial in changing environments)	+ Adaptations to changing environments are promoted through mechanisms such as bet-hedging and increased genetic diversity
Symbiont transmission and dispersal	Horizontal	+ Partner choice can take effect: good symbionts can be chosen, bad symbionts can be avoided or replaced	+ Competition with close relatives can be avoided + Escape from the host is possible
	Vertical	+ Ensures having a (good) symbiont in the next generation (partner fidelity) + Host/symbiont co-evolution can occur, increasing productivity	+ Ensures having a host in the next generation + Host/symbiont co-evolution can occur, increasing productivity
Symbiont diversity	Monoculture	+ Increased productivity through uniform farming conditions and potential for effective artificial selection + Harmful competition between symbionts is avoided	+ Symbionts that are consumed by their host gain indirect fitness benefits via dispersing/reproducing relatives
	Polyculture	+ Increased productivity through increased diversity (diversity-productivity relationship) + Symbiont community is more resilient against specialized parasites	+ Competition with close relatives is avoided + Opportunity to outcompete other symbionts

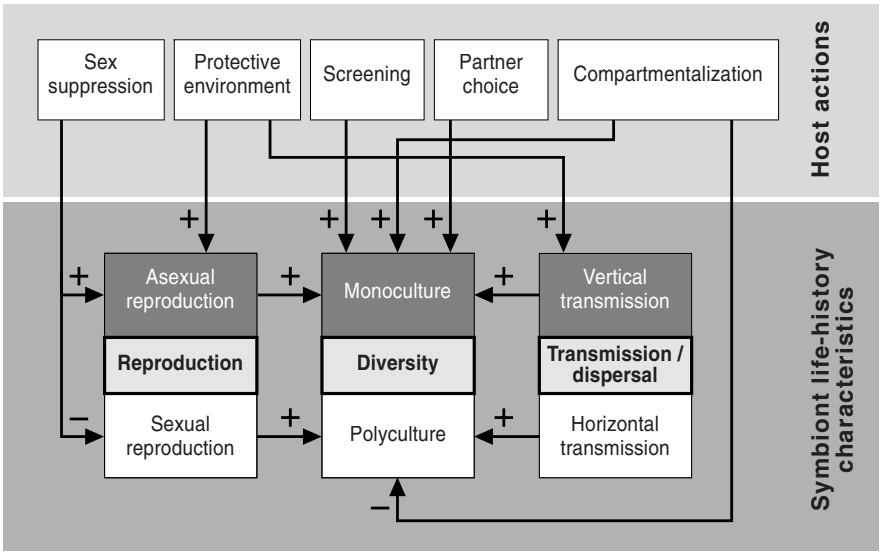


Figure 1.1 Flow diagram of host actions and symbiont traits involved in farming mutualism conflict resolution. The top panel lists host actions, the lower panel lists symbiont life-history characteristics and their arenas of potential conflict with their possible outcomes (symbiont reproduction, diversity, transmission). Two sets of outcomes are often observed in nature: asexual reproduction, monoculture and vertical transmission (in grey) and sexual reproduction, polyculture and horizontal transmission. Arrows show relationships and plus and minus signs indicate the nature of the relationship (positive or negative).

performance (Kiers & Denison 2008; Weyl *et al.* 2010). The relative importance of these mechanisms is somewhat controversial, but available data suggest that monocultures are commonly found in the farming mutualisms that have been studied, from the gardens of algae-growing damselfish (Hata & Kato 2002) to those of fungus-growing termites and ants (Bot *et al.* 2001; Poulsen & Boomsma 2005; Aanen *et al.* 2009; Mueller *et al.* 2010). In fungus-farming leaf-cutting ants, monocultures appear to be enforced by a combination of incompatibility between genetically different symbiont strains and active symbiont policing by the hosts (Poulsen & Boomsma 2005; Ivens *et al.* 2009; Mueller *et al.* 2010), whereas a simple mechanism of positive frequency-dependent propagation within established colonies appears sufficient to enforce life-time commitment between a termite host colony and a single symbiont clone (Aanen *et al.* 2009). However, more studies are needed to establish the generality of this principle, particularly for farming mutualisms where hosts are able to segregate symbionts in space (*compartmentalization*) or time to avoid competition (Palmer *et al.* 2010), so that the benefits of polyculture might surpass the costs.

What becomes clear from the sections above is that these three arenas of potential conflicts and their possible outcomes are not independent and all three are closely intertwined in a complicated web of costs and benefits of possible outcomes, host control mechanisms and symbiont life-history characteristics (see Table 1.2, Figure 1.1). Figure 1.1 shows how

the alternative outcomes of the three conflicts are influenced directly and indirectly by mechanisms of host control (top panel) and interactions among each other (lower panel). In practice, two sets of outcomes are often found in nature: (1) asexual reproduction, monocultures and vertical transmission (given in grey) vs. (2) sexual reproduction, polycultures and horizontal transmission.

L. FLAVUS ANTS FARMING ROOT APHIDS – A CASE STUDY

The study system

The focal empirical study system of this thesis is the interaction between the Yellow meadow ant *Lasius flavus* and the root aphids it tends in underground nests. This subterranean interaction is a typical example of a farming mutualism as the ants actively tend, house and protect the root aphids on which they depend for sugar (through honeydew consumption) (Figure 1.2) and nitrogen (through aphid consumption) (Pontin 1958; 1961b; 1978; Heie 1980; Seifert 2007). Also the aphids are presumed to be obligately dependent on this interaction as they presumably cannot survive without the grooming and the protective environment offered by the ants (personal observation, Pontin 1959; Paul 1977; Heie 1980; Hölldobler & Wilson 1990; Smart 1991). Most aphid species found in nests of *Lasius flavus* show myrmecophilous adaptations (adapted to living with ants) that preclude a free-living lifestyle (Paul 1977; Heie 1980; Hölldobler & Wilson 1990) and aphid population growth has previously been reported to increase with ant attendance (Smart 1991).

Although *Lasius flavus* and its root aphids are commonly found in Northwest Europe and the mutualistic character of the interaction has been known for decades [with the earliest reference describing aphids from ant nests dating from 1738 (Réaumur 1738)], the system has seldom been studied and most natural history characteristics remain unknown. For example, it has remained unclear how new ant colonies acquire aphids, how (genetically) diverse aphid life stocks are, how these aphids reproduce, and what the costs and benefits for the mutualistic partners are. Consequently, our knowledge of this study system at the beginning of the project was very limited and based on a sparse set of literature, mainly consisting of exploratory studies by Pontin in the late 60's and 70's, aphid

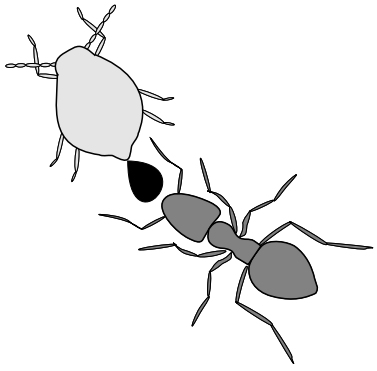


Figure 1.2 Schematic view of *Lasius flavus* worker 'milking' a root aphid for honeydew.

Figure 1.3 Impressions from the field. A) aphid chamber with aphid (*Geoica utricularia*) livestock, B) salt marsh with *Lasius flavus* mounds, C) sampling aphids from an ant mound (photos by: Aniek Ivens (A, B), Thijs Janzen (C)).

records by Heie (1980), two British dissertations (Paul 1977; Smart 1991) and two Danish studies from the early 90's (Godske 1991; 1992). Below, I will describe the 'players' of the mutualism in more detail, as well as the scope for conflict and cooperation in this mutualism.

The host ants

Lasius flavus is an ant species that occurs throughout the Palearctic region. It is quite common in Northwest and Central Europe, where it mostly occurs in stable (chalk-) grasslands and on salt marshes. Its species name *flavus* refers to its yellow colour, which is caused by the lack of pigment resulting from its subterranean life style as compared to its darker 'cousin' *Lasius niger*. Especially in salt marsh habitats and old, extensively grazed, pastures *L. flavus* colonies build conspicuous mounds that can be elevated up to 50 cm above the soil surface and reach diameters of more than 120 cm (Figure 1.3). When not disturbed, these mounds can remain for more than 100 years, being re-colonized by new ant colonies over time. *L. flavus* populations can reach average densities that are among the highest reported for ants (Boomsma & Van Loon 1982). In the Netherlands, only a few of these high density populations remain, notably at old cow pasture 'Junner Koeland', near Ommen and on the salt marsh of the island of Schiermonnikoog. Most empirical research presented in this thesis was performed at this latter location (Chapter 2; 4; Box A).

L. flavus ants appear to live in colonies with a single or at most a few queens (mono – and polygynous colonies) and colony size can reach numbers of 23000–100000 workers (Boomsma *et al.* 1993; Seifert 2007). Colonies are generally monodomous, *i.e.* separate nest mounds belong to separate colonies. Mating takes place in mating flights when virgin queens and males leave their natal nest to meet in the air on a single or very few suitable days in the summer. Queens mate 1–2 times and found a new colony after shedding their wings (Boomsma *et al.* 1993; Janzen 2009; van Boheemen 2010). Queens have also been reported to peacefully co-found new colonies. Queens can live up to 22.5 years (Boomsma *et al.* 1993; Seifert 2007).

The soil nests of *L. flavus* contain especially constructed aphid chambers (Figure 1.3A), where the ants actively protect and maintain their aphid livestock and protect against

parasites and predators (Pontin 1958; 1959; 1961b; Paul 1977; Pontin 1978). The honeydew secreted by the aphids is likely to be the almost exclusive source of carbohydrates for the fully subterranean *L. flavus* ants, as other subterranean carbon sources are very limited in the typical salt marsh and chalk-grassland habitats of these ants. Stable isotope analyses have confirmed that in the presence of aphids there is a demonstrable intermediate trophic level between the vegetation and the ants, consistent with the aphids being an intermediate food web node (A.B.F. Ivens & M. Schrama, unpublished data). *L. flavus* ants have also been recorded to eat a major proportion of their root aphids, suggesting that they also utilize their 'dairy cattle' to increase protein acquisition (Pontin 1958; 1961b; 1978; Smart 1991; Ivens *et al.* 2012a; Chapter 4). This implies that, similar to other subterranean ants, *L. flavus* depends on root aphid husbandry for producing colonies large enough to reproduce (Way 1963; Hölldobler & Wilson 1990).

The symbiont aphids

Thirteen different species of root aphids have been frequently described from *Lasius flavus* nests (Pontin 1978; Heie 1980; Godske 1991; Chapter 8) although some records mention even 23 species (Paul 1977; Seifert 2007). These thirteen species vary substantially in their abundance, distribution and (known) natural history details (Pontin 1978; Heie 1980; Godske 1991; 1992). This made us decide to focus empirical studies on the four most common and abundant species at our study site Schiermonnikoog: *Geoica utricularia*, *Tetraneura ulmi*, *Forda marginata* and its sibling species *Forda formicaria*.

These four root aphid species appear to be obligately dependent on living with ants (*i.e.* to be obligate myrmecophiles), as they have all lost predator defence mechanisms common in other aphids (Way 1963; Paul 1977; Heie 1980; Hölldobler & Wilson 1990): All four species have poorly developed cornicles (organs for protective wax production) and none of them is able to jump (Way 1963; Paul 1977; Heie 1980) as free living species typically do to escape predation. Most importantly, they all have a set of long anal hairs that can hold droplets of honeydew, a 'trophobiotic organ' that is only found in myrmecophilous aphids (Way 1963; Paul 1977; Heie 1980; Hölldobler & Wilson 1990). These aphids are most often associated with *L. flavus*, but can also be found in nests of other ant genera, such as *Myrmica* (Heie 1980). Apart from having distinct myrmecophilous traits that reveal obligate dependence on ant care, some of these aphids are also dependent on the ants for survival during overwintering (Way 1963; Paul 1977; Heie 1980). The sparse literature suggests that there may be considerable variation in this overwintering adaptation, as some aphids are known to have maintained holocyclic sexual reproduction (see box on aphid reproduction) and would therefore have to leave the ant nests during autumn, whereas others are facultatively or obligately anholocyclic (Paul 1977; Pontin 1978; Heie 1980; Godske 1991), *i.e.* do not move host plant and are permanently parthenogenetic. This possible polymorphism provided a main incentive for choosing these four focal species in our studies, as differences in reproductive cycle would provide interesting test beds for theory on potential host-symbiont conflict over symbiont reproductive mode. It also remains unclear how the root aphids and the ants embark on their colony specific associations. In a pilot study (Box A of this thesis), I therefore investigated the ability of ants to discriminate between different aphid species and genetic lineages.

POTENTIAL FOR CONFLICT AND COOPERATION IN THE *LASIUS FLAVUS* - ROOT APHID SYSTEM

Aphid reproduction

The fact that the root aphids tended by *Lasius flavus* may have the ability to reproduce both sexually and asexually (see box on aphid reproduction) implies a potential conflict of reproductive interest between the ants and the aphids over the best aphid reproductive mode. Holocyclic sexual reproduction that requires the aphids to leave the ant nest yields the advantages associated with sexual reproduction for the aphid symbiont, but is almost certainly not in the interest of the ants as it would make them lose their livestock partners. On the other hand, a stable environment might render risky sexual reproduction unnecessary for the aphids, whereas the ants might prefer at least some sexual reproduction among their aphids, for example to increase aphid resilience against parasites. Data on this conflict and its resolution are very sparse. Ant suppression of aphid sex has not been reported, although several mechanisms of suppressing aphid dispersal by other host ants (delaying alate (winged individuals) production, active wing clipping) may indicate the potential of host ant control over aphid sexual reproduction by prohibiting aphids to disperse to the primary host plant for sexual reproduction (Way 1963; Hölldobler & Wilson 1990). In addition, many above-ground myrmecophilous aphids are known to have maintained their sexual cycle (Heie 1980; Yao 2010; Vantaux *et al.* 2011a), but reproduction of myrmecophilous root aphids had not been studied in detail before I embarked on my thesis project.

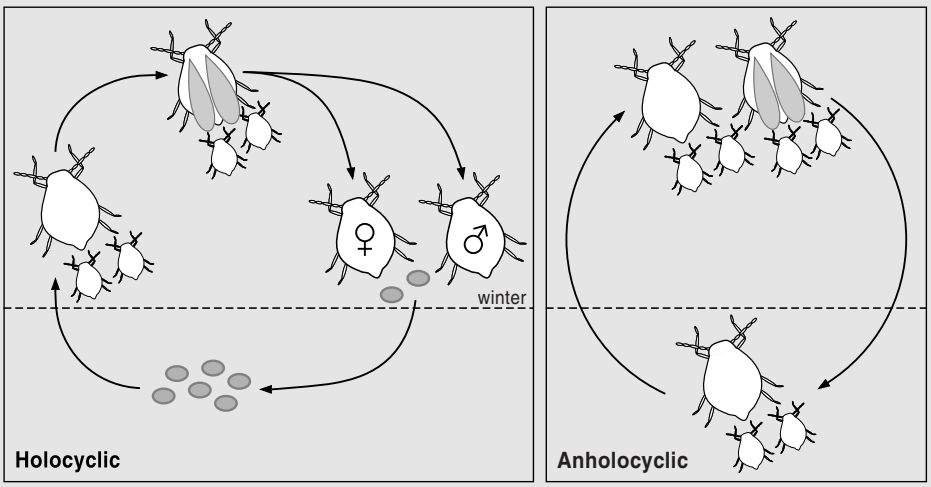
The sparse available literature suggests that all four focal aphid species have been observed to live anholocyclically on the roots of secondary host grasses (*Festuca rubra*, *Agrostis* spp. and *Elytrigia maritima*) in ant mounds (Muir 1959; Pontin 1978; Heie 1980; Godske 1991; Godske 1992). The *Forda* species and *G. utricularia* appear to be anholocyclic in Northern and Central Europe (including the sampling area of our study), but holocyclic in Southern Europe, where their primary host *Pistacia* spp. (Anacardiaceae) occurs (Heie 1980). In contrast to the other three species, *T. ulmi* has been observed to be holocyclic at several sites in Northwest Europe and Scandinavia (Heie 1980). This species was therefore expected to also be holocyclic in The Netherlands, with an obligate annual host shift to elm trees (*Ulmus* spp.; Ulmaceae) as primary host for sexual reproduction in autumn (Heie 1980). However, Pontin (1978) and Godske (1991) suggested that *T. ulmi* can also abandon its sexual phase and occur anholocyclically on grass roots in Northwest Europe. This indicated that there might be substantial intra- and interspecific variation in reproductive mode and life-cycle, suggesting different outcomes within a single system for this potential host-symbiont conflict. This formed the main incentive for a detailed study of root aphid reproductive mode with the use of genetic markers (Ivens *et al.* 2011; 2012b; Chapters 2; 3; 5).

Aphid dispersal

A second potential conflict between *L. flavus* and its root aphids concerns aphid dispersal, as there is both conflict of interest on transmission *mode* (horizontal vs. vertical transmission) and, in case of horizontal transmission, over the *frequency* of dispersal. Observation

Aphid reproduction

Aphids have two alternative and partly overlapping reproductive cycles (see figure): (1) in the case of holocyclic reproduction, aphids reproduce asexually during most of the year, with a single sexual generation in a distinct season, *e.g.* just before overwintering. This form of cyclic asexuality can involve obligate dispersal to a primary host plant, on which mating occurs, and the recurrent colonization of a secondary host plant for asexual reproduction during the rest of the year. (2) In the case of anholocyclic reproduction, aphids reproduce exclusively asexually. Anholocyclic aphids often live on a secondary host plant year around, because the primary host of their close sexual relative is no longer used. Closely related aphid species may have very different reproductive cycles (reviewed in Simon *et al.* 2002), and even populations of the same species have been found to differ in their mode of reproduction (Heie 1980; Simon *et al.* 1996; Delmotte *et al.* 2002; Gilabert *et al.* 2009; Kanbe & Akimoto 2009).



Schematic diagram of aphid life cycles. The left panel shows a holocyclic cycle, in which aphids reproduce clonally through the year and sexually in autumn, after which the eggs overwinter. The right panel shows an anholocyclic cycle in which aphids reproduce clonally throughout the year. Note that winged dispersers can occur in both cycles. Figure modified from Heie 1980.

of vertical transmission (vectoring) by ant queens during mating flights has been restricted to some highly specialized interactions that involve coccids (Way 1963; Hölldobler & Wilson 1990), so that aphids or coccids are assumed to be horizontally transmitted in other ant-homopteran interactions. However, ants have repeatedly been shown to restrict aphid dispersal. Indirect evidence for restricted dispersal was obtained by Yao (2010) after finding higher F_{IS} values for (holocyclic) ant-attended *Tuberculatus* aphids than for unattended (holocyclic) aphids. More direct evidence comes from observations of delayed

aphid dispersal and lower alate production in various ant-aphid interactions (Way 1963; Kindlmann *et al.* 2007; Oliver *et al.* 2007; Yao 2010; Tegelaar *et al.* 2011). The underlying mechanisms described for *Lasius* ants vary from dispersal inhibition through semiochemicals that slow down aphids (Oliver *et al.* 2007) to population regulation preventing aphid numbers reaching the critical threshold for alate production (Way 1963), and wing-clipping behaviour. Alate (winged) individuals have been described for the four most common (and focal) aphid species in *L. flavus* nests in at least some populations (Paul 1977; Heie 1980). These could either be asexually produced dispersers or, where these winged individuals were produced in connection with a host shift, be indicators of sexual reproduction not having been lost completely. Alate frequency and population viscosity of these four species were further investigated in my study presented in Chapter 2, to gain more insight into the outcome of the potential conflict on aphid transmission and dispersal.

Aphid (genetic) diversity

The third and last potential conflict between the ants and their root aphids concerns aphid livestock diversity. For the ants, a homogenous aphid livestock will prevent the potential costs of virulent aphid-aphid competition and thus increase productivity of the system. On the other hand a diverse aphid livestock might be more resilient against specialized pathogens. Aphids may also gain from living in a polyculture, because competition with close relatives is reduced when there is at least some niche segregation (*e.g.* using different grass species). Clonal mixing has been well-studied in social gall-forming aphids (among others Abbot 2009). In this context, increased relatedness among members of an aphid colony is predicted to promote cooperation between the gall members. These studies showed that relatedness among gall members tended to be high, but clonal diversity increased over the course of the season, as dispersing aphids entered existing colonies. Similar results were obtained by Vantaux *et al.* (2011a) for tree-dwelling, ant-tended, aphids. This study also showed that ant-tending did not affect aphid colony diversity, confirming earlier findings by Yao & Akimoto (2009). No previous studies have addressed the details of within-nest root aphid genetic- and species diversity. The molecular markers developed in my studies (Ivens *et al.* 2011; Chapter 5) enabled me to address these issues in detail in the study presented in Ivens *et al.* (2012a; Chapter 4).

OUTLINE OF THE THESIS

In this thesis, I will first present my empirical work after which I will continue with the more general theoretical chapters to conclude with an epilogue that focusses specifically on farming mutualisms. In **Chapter 2,** I investigated outcomes of the first two potential host-symbiont conflicts on symbiont reproductive mode and dispersal in the *L. flavus* system. Using microsatellite markers, I show that the four most common root aphid species in *L. flavus* nests reproduce predominantly clonally and that dispersal is rare in all species. In **Chapter 3**, I reflect on the statistical methods available for determining reproductive mode from microsatellite data, as applied in Chapter 2. This simulation study using the population genetic data of Chapter 2 shows that strict asexual reproduction is hard to

confirm with traditional statistical methods based on microsatellite data only. **Chapter 4** then focusses on the outcomes with respect to symbiont diversity. In this chapter, I investigated aphid (genetic) diversity within *L. flavus* mounds, showing that different aphid species and clonal lineages occur mostly spatially separated in *Lasius flavus* nests. In addition, the results indicate that the study system may be an example case of prudent husbandry for both carbohydrates and proteins. Whether ants are able to actively discriminate between different aphids, a prerequisite for complete host control over symbionts, remains unclear. **Box A** reports on a pilot study in which I conducted partner choice experiments to investigate ant discriminatory ability between different aphids. **Chapter 5** reports on the development and cross-amplification of the microsatellite markers used in Chapters 2, 3, 4 and Box 1 and similar markers developed for three additional root aphid species commonly found in nests of *Lasius flavus*. To conclude the empirical part of the dissertation, **Box B** reports on the software to detect clonal lineages in a microsatellite dataset, which I updated to use in Chapter 2. With **Chapter 6** the theoretical part of this thesis starts. This modelling study aimed to investigate the joint evolution partner fidelity, partner choice and within-species cooperation. I show that conditional dispersal (equivalent to partner fidelity) can indeed promote the evolution of cooperation, but that conditional settlement (partner choice) only plays a marginal role in this model. Also, the evolved conditional strategies differ strikingly from what is generally expected in this context. In **Chapter 7** I present a follow-up study to Chapter 6 to investigate whether the same results apply to between-species cooperation. The study shows that in this case both conditional dispersal and conditional settlement promote the evolution of cooperation. Also, I show that these processes can lead to asymmetric cooperative interactions that are highly dynamic. I will conclude with an epilogue presented in **Chapter 8**, which will focus on the new insights into farming mutualism gained from this thesis, as well as an evaluation of the study system and discussion of aspects of the *L. flavus* system that have not been treated in this dissertation. I will also offer some thoughts on promising future research avenues in the study of the evolutionary ecology of (farming) mutualisms.

CHAPTER 2

Reproduction and dispersal in an ant-associated root aphid community

Aniek B.F. Ivens
Daniel J.C. Kronauer
Ido Pen
Franz J. Weissing
Jacobus J. Boomsma

Published in *Molecular Ecology*: 2012, **21**, 4257–4269

ABSTRACT

Clonal organisms with occasional sex are important for our general understanding of the costs and benefits that maintain sexual reproduction. Cyclically parthenogenetic aphids are highly variable in their frequency of sexual reproduction. However, studies have mostly focused on free-living aphids above ground, whereas dispersal constraints and dependence on ant-tending may differentially affect the costs and benefits of sex in subterranean aphids. Here, we studied reproductive mode and dispersal in a community of root aphids that are obligately associated with the ant *Lasius flavus*. We assessed the genetic population structure of four species (*Geoica utricularia*, *Tetraneura ulmi*, *Forda marginata* and *F. formicaria*) in a Dutch population and found that all species reproduce predominantly if not exclusively asexually, so that populations consist of multiple clonal lineages. We show that population viscosity is high and winged aphids rare, consistent with infrequent horizontal transmission between ant host colonies. The absence of the primary host shrub (*Pistacia*) may explain the absence of sex in three of the studied species, but elm trees (*Ulmus*) that are primary hosts of the fourth species (*T. ulmi*), occurred within a few km of the study population. We discuss the extent to which obligate ant-tending and absence of primary hosts may have affected selection for permanent parthenogenesis and we highlight the need for further study of these aphids in Southern Europe where primary hosts may occur close to *Lasius flavus* populations, so that all four root aphid species would have realistic opportunities for completing their sexual life-cycle.

INTRODUCTION

Asexual reproduction can allow rapid population growth and therefore enable quick colonization of new habitats. Nevertheless, exclusive asexual reproduction is generally considered an evolutionary 'dead end' because prolonged lack of recombination will lead to accumulation of deleterious mutations and slower evolvability in response to parasites and environmental change, ultimately driving asexual species to extinction. Occasional sex in an otherwise asexual species, however, can allow for sufficient recombination to outweigh these costs, without compromising the benefits of asexual reproduction (a.o. Green & Noakes 1995, reviewed in D'Souza & Michiels 2010). Studying species with such mixed reproductive modes can thus enhance our understanding of the evolution and maintenance of sex, one of the major current topics in evolutionary biology.

Aphids are particularly informative in this context, because they are often cyclical parthenogens with a (holocyclic) reproductive cycle consisting of a sequence of asexual generations followed by a single sexual generation (Simon *et al.* 2002). However, some aphids have lost the sexual generation and reproduce exclusively asexually (anholocyclic). These variable reproductive modes, both between species and between populations of the same species, make aphids excellent model systems for studying the selective forces that affect cyclical parthenogenesis (Simon *et al.* 1996; 1999; Delmotte *et al.* 2002; Gilabert *et al.* 2009; Kanbe & Akimoto 2009; Vantaux *et al.* 2011a).

Aphids are also well-known for their mutualistic relationships with ants (Way 1963; Stadler & Dixon 2008), which can have profound effects on dispersal and reproduction, and thus on the population structure of mutualistic partners (Herre *et al.* 1999; Leigh 2010). However, most of the previous aphid studies have focused on non-myrmecophiles in above-ground populations, whereas subterranean and obligately ant-associated aphids have been neglected (Yao & Akimoto 2009; Yao 2010). Here, we focus on four sympatric species of root aphids (*Geoica utricularia*, *Tetraneura ulmi*, *Forda marginata*, *F. formicaria*) that are known to be obligately associated with ants (Heie 1980; Seifert 2007).

The specific objectives of our study were: (1) to infer the extent of population viscosity of multiple root aphid species across a field transect spanning seven km (Figure 2.1), (2) to determine the dominant mode of reproduction in these aphids, and (3) to estimate the potential for horizontal dispersal via winged forms.

MATERIALS AND METHODS

Root aphid natural history

Aphids have two alternative and partly overlapping reproductive cycles: (1) Holocyclic reproduction where aphids are propagated asexually during most of the year, but have a single sexual generation in a distinct season often just before overwintering. This form of cyclic asexuality can involve obligate dispersal to a primary host plant, on which mating occurs, and the recurrent colonization of a secondary host plant for asexual reproduction during the rest of the year (Heie 1980; Simon *et al.* 2002). (2) Anholocyclic reproduction where aphids are propagated by obligate parthenogenesis. These aphids often live on a

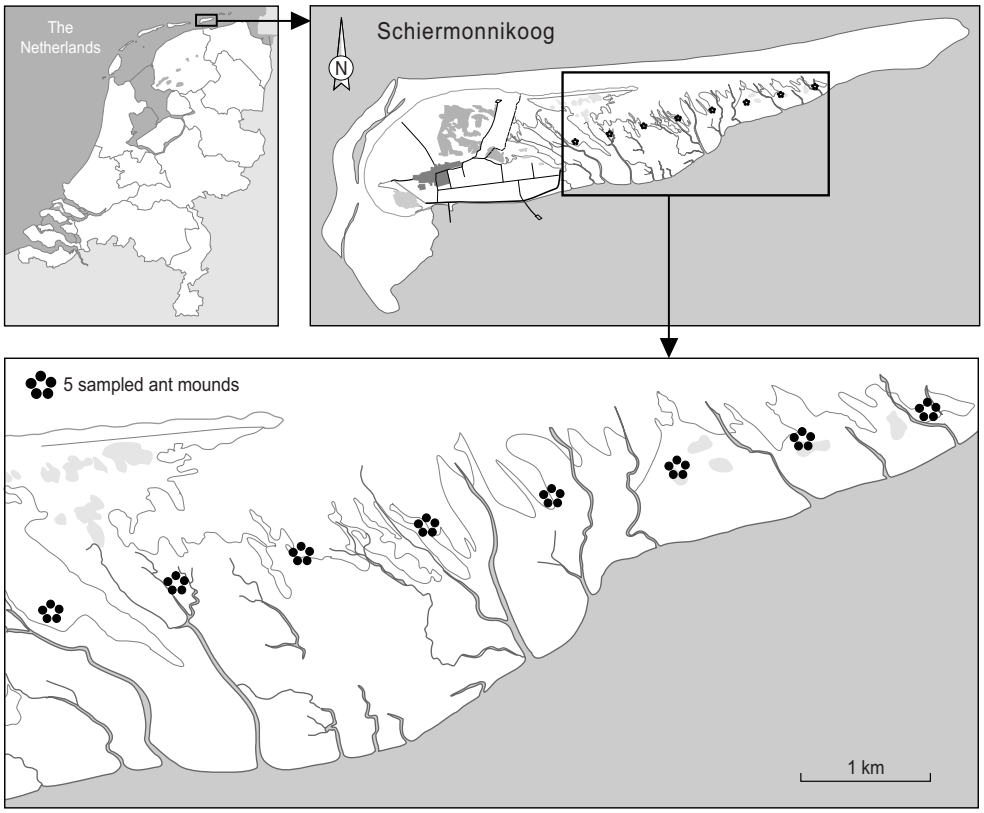


Figure 2.1 Sampling site of root aphids associated with *Lasius flavus* mounds. Aphids were collected on the island of Schiermonnikoog (The Netherlands), where ant mounds were sampled in groups of five along a 7 km transect across the salt marsh of the island (maps courtesy of D. Visser).

secondary host plant year-round, because the primary host of their close sexual relative is no longer used. Closely related aphid species may have very different reproductive cycles (Simon *et al.* 2002), and even populations of the same species have been found to differ in their mode of reproduction (Heie 1980; Simon *et al.* 1996; Delmotte *et al.* 2002; Gilabert *et al.* 2009; Kanbe & Akimoto 2009).

The sparse available literature suggests that all four focal aphid species of this study (*Geoica utricularia*, *Tetraneura ulmi*, *Forda marginata* and *F. formicaria*) have been observed to live anholocyclically on the roots of secondary host grasses (*Festuca rubra*, *Agrostis* spp. and *Elytrigia maritima*) inside ant mounds (Muir 1959; Pontin 1978; Heie 1980; Godske 1991; 1992). The *Forda* species and *G. utricularia* appear to be anholocyclic in Northern and Central Europe (including our study site on the Dutch island of Schiermonnikoog), but holocyclic in Southern Europe, where their primary host *Pistacia* spp. (Anacardiaceae) occurs (Heie 1980). In contrast to the other three species, *T. ulmi* has been observed to be holocyclic at several sites in Northwestern Europe and Scandinavia

(Heie 1980). This species was therefore expected to be holocyclic in our sampling area, with an obligate annual host shift to elm trees (*Ulmus* spp.; Ulmaceae) as primary host for sexual reproduction in autumn (Heie 1980). However, Pontin (1978), Godske (1991) and Blackman & Eastop (1994) suggested that *T. ulmi* can also abandon its sexual phase and occurr anholocyclically on grass roots in Northwestern Europe. Alate (winged) individuals have been described for all four species in at least some populations (Paul 1977; Heie 1980). These could either be indicators of sexual reproduction and host shift not having been lost completely, or asexually produced dispersers, although the existence of the latter remains unconfirmed in Pemphigidae (Moran 1992). We therefore started this study with the hypothesis that there might be intra- and interspecific variation in reproductive mode and life cycle among the root aphids in our focal Dutch population.

All four aphid species are found in soil nests of the ant *Lasius flavus*. These nests contain specifically constructed aphid chambers, where the ants actively protect their livestock from parasites and predators (Pontin 1959; 1978), and where they both tend and eat them (Pontin 1958; 1961b; 1978). All four root aphid species appear to be obligate myrmecophiles, having lost predator defense mechanisms common in other aphids (Way 1963; Paul 1977; Heie 1980; Hölldobler & Wilson 1990), *i.e.* all have poorly developed cornicles (organs for protective wax production) and lack saltatorial legs to jump or actively drop from branches in response to threats (Way 1963; Paul 1977; Heie 1980). Most importantly, they all have a set of long anal hairs that can hold droplets of honeydew, a 'trophobiotic organ' that is only found in myrmecophilous aphids (Way 1963; Heie 1980; Hölldobler & Wilson 1990) (Figure 2.2). Apart from these distinct traits that reveal obligate dependence on ant care, some of these aphids are also dependent on the ants for survival during overwintering (Way 1963; Heie 1980). However, the sparse literature suggests that there may be considerable variation in this overwintering adaptation, as some aphids are known to have maintained holocyclic sexual reproduction (see above) and would therefore have to leave the ant nests during autumn, whereas others are facultatively or obligately anholocyclic (Pontin 1978; Heie 1980; Godske 1991). How ant colonies acquire their mutualistic aphids remains poorly understood, as virgin queens have not been observed to vector aphids during colony founding (A.B.F. Ivens, personal observation; Chapter 8). Most likely, ant mounds that have recently gone extinct are recolonized by founding queens or neighbouring smaller colonies so that local aphid lineages may acquire new 'owners' (Ivens *et al.* 2012a; Chapter 4). In addition, aphids may disperse independently by wind, walking or floating on tidal water (Foster & Treherne 1978; Foster 1978).

Sampling

All aphid samples were collected in July 2008 from *L. flavus* ant mounds on the island of Schiermonnikoog, the Netherlands (53°28' N, 6°09' E). Sampling followed a 7 km transect across the salt-marsh of the island (Figure 2.1), with the westernmost kilometer of the transect located on grazed pasture and the remainder on un-grazed salt-marsh. The transect was subdivided into eight locations with 1 km intervals. Nest mound density was high throughout the transect (ca. 600–3500 mounds per ha). At each location, soil samples of five ant mounds of similar size (Ø ca. 80 cm) were taken and hand-sorted for the occurrence of wingless and winged (alate) root aphids. The precise location of each ant nest was

recorded with a GPS device (eTrexVista™, Garmin, 0.5–5 m precision). A subsample of all collected aphids was used for species identification using a protocol for microscopic preparation modified after (Heie 1980). Reference specimens are located at the University of Groningen (access available upon request).

We chose to use small soil cores so that sampling was non-destructive and we could resample mounds in later years. This sampling scheme was nonetheless sufficient to obtain at least one aphid belonging to each of the focal species from 30 of the 40 mounds, and only one of the 40 mounds yielded no aphids at all in any of our soil core samples. All mounds were confirmed to be inhabited by *L. flavus*, with ca. 60% of the 21 soil cores taken per mound containing ants.

Molecular analysis

DNA was extracted from entire aphids using 200 μl 20%-Chelex® 100 resin (Fluka) (Walsh *et al.* 1991). We used polymorphic microsatellite markers to genotype specimens of *Forda* spp. (loci Fm1, Fm3, Fm4, Fm6, Gu6, Gu11, Gu13), *G. utricularia* (loci Gu2, Gu3, Gu5, Gu6, Gu8, Gu9, Gu11, Gu13), and *T. ulmi* (loci Tu1, Tu2, Tu3, Tu4, Tu10, Tu11). Marker-specific details and amplification protocols are given in Ivens *et al.* (2011; Chapter 5). If a marker failed to amplify in an individual, the amplification process was repeated at least twice. PCR-products were analyzed on an ABI-PRISM 3130XL (Applied Biosystems) sequencer and chromatograms were analyzed in Genemapper (Applied Biosystems).

Data analysis

After omitting 30 samples in which more than half of the markers repeatedly failed to amplify, the total number of individuals included in the datasets was 469: 201 for *G. utricularia*, 92 for *T. ulmi*, 158 for *F. marginata* and 18 for F. *formicaria*.

MLGsim 2.0 (http://www.rug.nl/fmns-research/theobio/downloads), an updated version of MLGsim (Stenberg *et al.* 2003b; Box B), was used to group individuals into diploid multilocus genotypes (MLGs), *i.e.* unique combinations of alleles across all tested marker loci. Where missing data occurred (190 of the 469 individuals had some missing genetic marker data - on average 13% of the alleles was missing), individuals were joined into the MLG of which the genotype for the remaining successfully scored loci was identical. In the two cases where an individual matched two different but very similar MLGs at all successfully amplified loci, it was grouped with the more common MLG of the two. For our further analyses, gaps in an individual's MLG were filled in with the alleles of the MLG to which the individual had been assigned. While this increased sample size for our analyses, it also implied that we may have slightly underestimated overall genetic variability.

MLGs were either represented by several to many individuals (*recurrent MLGs*) or (rarely) as a single individual in our samples (*single MLGs*). Asexual reproduction will thus affect population genetic analyses through overrepresentation of clonally produced individuals (Sunnucks *et al.* 1997). To take any possible effects of pseudoreplication into account, all analyses were performed both on the full dataset, including all individuals (*ramet data*; *i.e.* all individuals belonging to a single MLG), and on a subset of the data consisting of only one individual per MLG (*genet data*) (*sensu* Harper 1977). The two analyses yielded comparable qualitative conclusions.

Estimates of genetic variability

Asexual reproduction tends to decrease segregation of alleles within loci and recombination between loci. Over time, this leads to observed heterozygosities (H_O) differing from those expected under sexual outbreeding (H_E), and to deviations from Hardy-Weinberg equilibrium (HWE). Specifically, asexual reproduction can lead to heterozygote excess [*i.e.* a negative fixation index F_{IS} (Weir & Cockerham 1984; Balloux *et al.* 2003; Stoeckel *et al.* 2006)] through mutation accumulation in clonal lineages, also known as the 'Meselson effect' in ancient asexual lineages (Birky 1996; Welch & Meselson 2000; Halkett *et al.* 2005). Nevertheless, mechanisms such as mitotic recombination or occasional sex can also lead to heterozygote deficiency in clones, particularly when asexual lineages are not very old and stochastic effects determine whether clones happen to be homozygous or heterozygous at neutral markers (Birky 1996). Finally, the lack of recombination under asexual reproduction should lead to significant linkage disequilibrium (LD). GENEPOP 4.0 (Rousset 2008) was used to estimate H_O, H_E, F_{IS}, linkage disequilibrium (LD), and deviations from HWE, assuming a panmictic population with random mating.

As a measure for genetic diversity we used the *G/N* ratio P_d, where *N* is the sample size for a given aphid species and *G* is the number of distinct MLGs in a focal population (Ellstrand & Roose 1987; Dorken & Eckert 2001; Arnaud-Haond *et al.* 2007). We also estimated P_{sex} values for every observed MLG, with P_{sex} being defined as the probability of obtaining at least as many as the observed number of individuals belonging to a given MLG under the null hypothesis of sexual reproduction and population-wide random mating (Tibayrenc *et al.* 1990; Parks & Werth 1993; Young *et al.* 2002; Arnaud-Haond *et al.* 2007). The calculation of P_{sex} takes into account the observed frequencies of the alleles constituting the given MLG. A MLG with a low P_{sex} value therefore indicates that the multiple individuals observed for that MLG likely originated from clonal reproduction rather than sexual reproduction. Although P_{sex} calculation was initially developed to confirm strict asexuality, it can also be applied to cyclical parthenogens. This is because P_{sex} estimations are done for each unique MLG separately and since this is based on population-wide allele frequencies, any present genetic variation naturally enters the analysis, regardless its origin. Using MLGsim 2.0, we estimated P_{sex} values and derived P-values for these values using Monte Carlo resampling simulations of our study population under HWE with 1000 iterations (Box B).

Genetic distance

We calculated genetic distances between MLGs using the shared allele distance DAS (Jin & Chakraborty 1993) in POPULATIONS 1.2.30 (© 1999, Olivier Langella, CNRS UPR9034). Pairs of MLGs with relatively small genetic distances might belong to one multilocus lineage (MLL) (Arnaud-Haond *et al.* 2007), *i.e.* a grouping of MLGs that go back to the same sexual reproduction event and whose genetic differences are due to later mutations, asexual recombination, or, possibly, scoring errors. We considered two MLGs as being part of the same MLL when they only differed by one or two alleles over all markers combined. The frequency distribution of the genetic distances between our samples was bimodal, with genetic distances of one or two alleles constituting the first peak of this overall frequency distribution, so that we felt confident that this distinction captured reality rather

well. We also used a more formal approach of assessing MLL/MLG distinctions, based on the frequency distribution of genetic distances, following (Arnaud-Haond *et al.* 2007). This method was designed for MLL assignments based on at least ten times more MLGs than observed in the present study, but the results were very similar to those obtained by our original approach, leading to identical overall conclusions. After multiplying by the number of alleles that were compared for distance to transform proportional distances into absolute allele differences, the distance matrix was used to visualize the relationships between MLGs using minimum spanning MLG trees constructed in HapStar v 0.6 (Teacher & Griffiths 2011). The distance matrix was also used to construct Neighbour Joining (NJ) trees (appendix).

Spatial autocorrelation analyses

To assess whether genetically similar individuals tended to occur geographically close to each other, we estimated spatial autocorrelations in all four aphid species. Spatial autocorrelation analysis tests whether the matrix of pairwise genetic distances significantly correlates with the matrix of pairwise geographic distances between individuals (Euclidian distance based on GPS-coordinates). We used a Mantel test of matrix correspondence with 10^4 permutations to test for significant correlation between the matrices (Smouse *et al.* 1986).

The occurrence of spatial autocorrelation can be graphically illustrated using correlograms, in which the estimated matrix correlation coefficient *r* is plotted against geographical distance, subdivided in classes. We used seven classes (0-1, 1-2, 2-3, 3-4, 4-5, 5-6 and 7-8 km), representing the distances between the eight sampled locations along the transect. Following Smouse & Peakall (1999) and Peakall *et al.* (2003), the estimated *r* value was plotted in a spatial correlogram with error bars representing the 95% CIs for *r* determined by bootstrapping with 10^4 replicates. The estimated *r* values were plotted alongside the 95% CIs of the *r*-values that were expected under the null hypothesis of a random distribution of individuals over locations (10^4 permutations). When an estimated *r* was larger than 0 and its 95% CI bars fell outside the 95% CIs generated by the null hypothesis, genetic and geographical distance were inferred to be positively correlated, with the first x-axis intercept representing the distance over which significant spatial structure occurred (Smouse & Peakall 1999; Peakall *et al.* 2003). All spatial autocorrelation analyses were performed in GENALEX 6.2 (Peakall & Smouse 2006) and correlograms were drawn using R 2.12.0 and the xyplot() function in the lattice package (Sarkar 2008).

RESULTS

Aphid genetic variability and reproduction

All four root aphid species showed significant deviations from Hardy-Weinberg Equilibrium (HWE) both at the *ramet* and the *genet*-level (Table 2.1). Most of the significant differences from HWE were caused by heterozygote deficiency, but some loci showed heterozygote excess and thus negative F_{IS} values (Table 2.1). In *G. utricularia*, significant *ramet*-level LD ($P < 0.05$) was present in all 28 pairs of loci, and at the *genet*–level in 17 of the 28 pairs.

In *T. ulmi*, *ramet*-level LD was detected in 11 pairs with only four loci-pairs showing no LD, whereas no significant LD was found at the *genet*-level. *F. marginata* showed *ramet*-level LD in all 23 pairs of loci and *genet*-level LD in 16 of the 23 pairs (Table 2.A1). In *F. formicaria*, only one pair of loci could be tested due to low sample sizes, showing no LD at either the *ramet*- or the *genet*-level. Combined with the consistent deviations from HWE, this predominance of LD is a strong indication for asexuality being the dominant mode of reproduction in three of the four species: *G. utricularia*, *T. ulmi* and *F. marginata*. Despite the low sample size, we infer that this is also likely to be the case for *F. formicaria*, because the biology of the two sibling species *F. marginata* and *F. formicaria* appears to be very similar (Heie 1980) and the observed allelic distribution over clonal lineages was similar as well (see below).

In total, we found nine MLGs among the 201 genotyped *G. utricularia* individuals. Three of these were unique 'single' MLGs, which could all be unambiguously grouped into MLLs with one of the six MLGs occurring in multiple copies (Table 2.2, Figure 2.2A, Figure 2.A1A). All MLGs with multiple samples had P_{sex} values significantly below expectation for random mating (Table 2.2). Genetic diversity P_d for this species was 0.044, with some MLGs being vastly overrepresented among the samples (Table 2.2). For example, the predominant *G. utricularia* MLG was found 120 times, *i.e.* in 60% of the samples (Table 2.2). Also these patterns indicate predominant clonal reproduction, and thus that this population of *G. utricularia* is anholocyclic. *T. ulmi* showed a similar pattern, with 4 MLLs consisting of 6 MLGs in total, all of which occurred more than once (Table 2.2, Figure 2.2B, Figure 2.A1B). The P_{sex} estimates for *T. ulmi* were also significantly reduced when tested against HWE expectation. In this species, the predominant MLG accounted for 52% of the samples and overall genetic diversity P_d was 0.065 (Table 2.2), indicating that *T. ulmi* is also anholocyclic and clonally propagated in the sampled population.

The same conclusion could be drawn for the two *Forda* species. *F. marginata* samples were more diverse than those of *G. utricularia* and *T. ulmi*, with a total of 18 MLGs, seven of which were encountered only once. All of these *single* MLGs and five of the *recurrent* MLGs could be combined with other MLGs into six MLLs (Table 2.2, Figure 2.2C, Figure 2.A1C). All but one of the P_{sex} values were significantly reduced, with the predominant clone accounting for 68% of the samples, and P_d being 0.114 (Table 2.2). Among the *F. formicaria* samples we found six MLGs, all belonging to a single MLL. In this species, P_{sex} values were also generally low, although mostly not significant, which appears to be due to low sample size. Most MLGs in this species were also recurrent, with 33% of the individuals belonging to the predominant clone, and P_d being 0.333 (Table 2.2). Interestingly, the *F. formicaria* MLGs formed a single clade within the *F. marginata* tree, confirming the close relationship between the two species and suggesting that further work is needed to unambiguously establish species identities in this group (Figure 2.2C, Figure 2.A1C).

Aphid dispersal

Figure 2.3 shows the relative frequencies of all detected MLGs and MLLs over the 7 km transect for *G. utricularia*, *F. marginata* and *T. ulmi*. Although all species had at least one MLG that was relatively abundant and distributed over more than two locations on the island (except for *F. marginata*), most MLGs only occurred at one or two sampled loca-

Table 2.1 Population genetic statistics for the four species of root aphids. Data are given both for the *ramet* (including all samples) and the *genet* level (excluding replicates of the same MLG), and for each tested locus and the population as a whole. *N* is the number of genotyped aphids, N_a the number of observed alleles, H_E and H_O are expected (under Hardy-Weinberg assumptions) and observed heterozygosities, and F_{IS} is the fixation index. Significant P-values for deviations from HWE (at the 0.2 % level after Bonferroni correction) are given in bold (see Materials and Methods).

		Ramet level					Genet level				
Locus	N_a	*N*	H_E	H_O	F_{IS}	HWE Pvalue	*N*	H_E	H_O	F_{IS}	HWE Pvalue
Geoica utricularia											
Gu2	5	201	0.58	0.31	0.465	**< 0.001**	9	0.63	0.22	0.680	**0.001**
Gu3	7	201	0.69	1.00	-0.457	**< 0.001**	9	0.79	1.00	-0.210	0.096
Gu5	6	201	0.57	0.14	0.746	**< 0.001**	9	0.77	0.33	0.607	**0.000**
Gu6	8	201	0.59	0.29	0.513	**< 0.001**	9	0.82	0.33	0.631	**< 0.001**
Gu8	4	201	0.55	0.06	0.892	**< 0.001**	9	0.72	0.22	0.719	**< 0.001**
Gu9	8	201	0.78	0.84	-0.068	**< 0.001**	9	0.66	0.33	0.539	**0.001**
Gu11	6	201	0.73	0.70	0.043	**< 0.001**	9	0.81	0.56	0.365	**< 0.001**
Gu13	8	201	0.78	0.90	-0.155	**< 0.001**	9	0.81	0.67	0.238	**0.002**
Population[1]	6.5	201	0.66	0.53	0.196		9	0.75	0.46	0.439	
Tetraneura ulmi											
Tu1	2	92	0.141	0.000	1.000	**< 0.001**	6	0.444	0.000	-0.053	0.031
Tu2	2	92	0.141	0.000	1.000	**< 0.001**	6	0.444	0.000	0.118	0.031
Tu3	5	92	0.702	0.924	-0.312	**< 0.001**	6	0.764	0.667	0.200	0.184
Tu4	5	92	0.621	0.902	-0.449	**< 0.001**	6	0.750	0.667	0.216	**0.001**
Tu10	3	92	0.281	0.326	-0.153	0.445	6	0.292	0.333	1.000	1.000
Tu11	4	92	0.384	0.402	-0.043	**0.001**	6	0.514	0.500	1.000	0.532
Population[1]	3.5	92	0.378	0.426	-0.120		6	0.535	0.361	0.404	
Forda marginata											
Fm1	9	155	0.45	0.25	0.442	**< 0.001**	16	0.79	0.56	0.320	**< 0.001**
Fm3	6	158	0.46	0.22	0.538	**< 0.001**	18	0.79	0.50	0.388	**< 0.001**
Fm4	6	158	0.43	0.15	0.665	**< 0.001**	18	0.70	0.28	0.622	**< 0.001**
Fm6	4	158	0.69	0.94	-0.353	**< 0.001**	18	0.73	0.78	-0.030	**< 0.001**
Gu6	5	158	0.68	0.71	-0.041	**< 0.001**	18	0.58	0.28	0.543	**< 0.001**
Gu11	6	158	0.46	0.21	0.548	**< 0.001**	18	0.79	0.44	0.460	**< 0.001**
Gu13	4	155	0.40	0.00	1.000	**< 0.001**	16	0.58	0.00	1.000	**< 0.001**
Population[1]	5.7	157	0.51	0.35	0.311		17	0.71	0.41	0.447	
Forda formicaria											
Fm3[2]	1	18	0.00	0.00		-	6	0.00	0.00		-
Fm4	3	18	0.50	0.78	-0.551	0.024	6	0.54	0.83	-0.471	0.638
Fm6	2	18	0.50	1.00	-1.000	**< 0.001**	6	0.50	1.00	-1.000	0.090
Gu6[2]	1	18	0.00	0.00		-	6	0.00	0.00		-
Gu11	3	18	0.44	0.28	0.393	0.072	6	0.57	0.33	0.487	0.226
Gu13[2]	1	18	0.00	0.00		-	6	0.00	0.00		-
Population[1]	1.8	18	0.24	0.34	-0.409		6	0.27	0.36	-0.262	

[1] Cumulative population-level tests of deviations from HWE would have been significant in all cases with the possible exception of the *F. formicaria* genets, but have not been performed because significant LD implies that locus-specific probabilities cannot be considered as independent.
[2] Loci that are monomorphic in *F. formicaria* and polymorphic in *F. marginata*. The alleles observed for Fm3 and Gu6 were diagnostic for *F. formicaria*.

Table 2.2 Multilocus Genotypes (MLG) of the four root aphid species. Name (clone acronym) and sample size (n) for each MLG; P_{sex}, the probability that the MLG occurred at least n times under the assumption of random mating is given for each *recurrent* MLG. All significant P_{sex}-values remained significant after Bonferroni correction with the exception of *F. formicaria* MLL1c. Total sample size (N), total number of MLGs (G) and genetic diversity P_d (G/N) are given for each species. Clone acronyms of MLGs consist of the species name abbreviation (Gu, Tu, Fm, Ff), the MLL-number (1-6), and a letter (a-f) that is unique for the MLG within its MLL.

MLG	*n*	P_{sex}
Geoica utricularia		
Gu-MLL1	17	0.000***
Gu-MLL2a	1	-
Gu-MLL2b	2	0.000***
Gu-MLL3a	1	-
Gu-MLL3b	11	0.000***
Gu-MLL4	46	0.000***
Gu-MLL5	2	0.000***
Gu-MLL6a	1	-
Gu-MLL6b	120	0.000***
N	**201**	
G	**9**	
P_d	**0.044**	

MLG	*n*	P_{sex}
Tetraneura ulmi		
Tu-MLL1a	48	0.000***
Tu-MLL1b	7	0.000***
Tu-MLL2a	3	0.000***
Tu-MLL2b	4	0.000***
Tu-MLL3	24	0.000***
Tu-MLL4	6	0.000***
N	**92**	
G	**6**	
P_d	**0.065**	

MLG	*n*	P_{sex}
Forda marginata		
Fm-MLL1a	1	-
Fm-MLL1b	1	-
Fm-MLL1c	1	-
Fm-MLL1d	2	0.009
Fm-MLL1e	108	0.000***
Fm-MLL1f	1	-
Fm-MLL2a	4	0.000***
Fm-MLL2b	5	0.000***
Fm-MLL2c	6	0.000***
Fm-MLL3a	5	0.000***
Fm-MLL3b	7	0.000***
Fm-MLL3c	3	0.000***
Fm-MLL3d	1	-
Fm-MLL4	2	0.000***
Fm-MLL5a	7	0.000***
Fm-MLL5b	1	-
Fm-MLL6a	1	-
Fm-MLL6b	2	0.000***
N	**158**	
G	**18**	
P_d	**0.114**	

MLG	*n*	P_{sex}
Forda formicaria		
Ff-MLL1a	1	-
Ff-MLL1b	4	0.072
Ff-MLL1c	6	0.009*
Ff-MLL1d	4	0.061
Ff-MLL1e	1	-
Ff-MLL1f	2	0.037
N	**18**	
G	**6**	
P_d	**0.333**	

*** P value of $P_{sex} < 0.001$
** P value of $P_{sex} < 0.01$
* P value of $P_{sex} < 0.05$

tions. Furthermore, the MLGs belonging to the same MLL tended to occur in a clustered manner (Figure 2.3), suggesting that dispersal levels are generally low. The Mantel test showed a significant correlation between genetic and geographic distances in *G. utricularia* and *F. marginata* (Figure 2.4AC). The correlograms were consistent with the outcome of the Mantel test, with significantly positive coefficients only being found for short (<3 km) distances in *G. utricularia* and *F. marginata*, with 95% confidence bars well above expectation based on a random distribution of genotypes over the island (Figure 2.4). No significant autocorrelation was found in the populations of the other species, *T. ulmi*. The low sample size for *F. formicaria* (n = 18) did not allow for conclusive estimation of dispersal

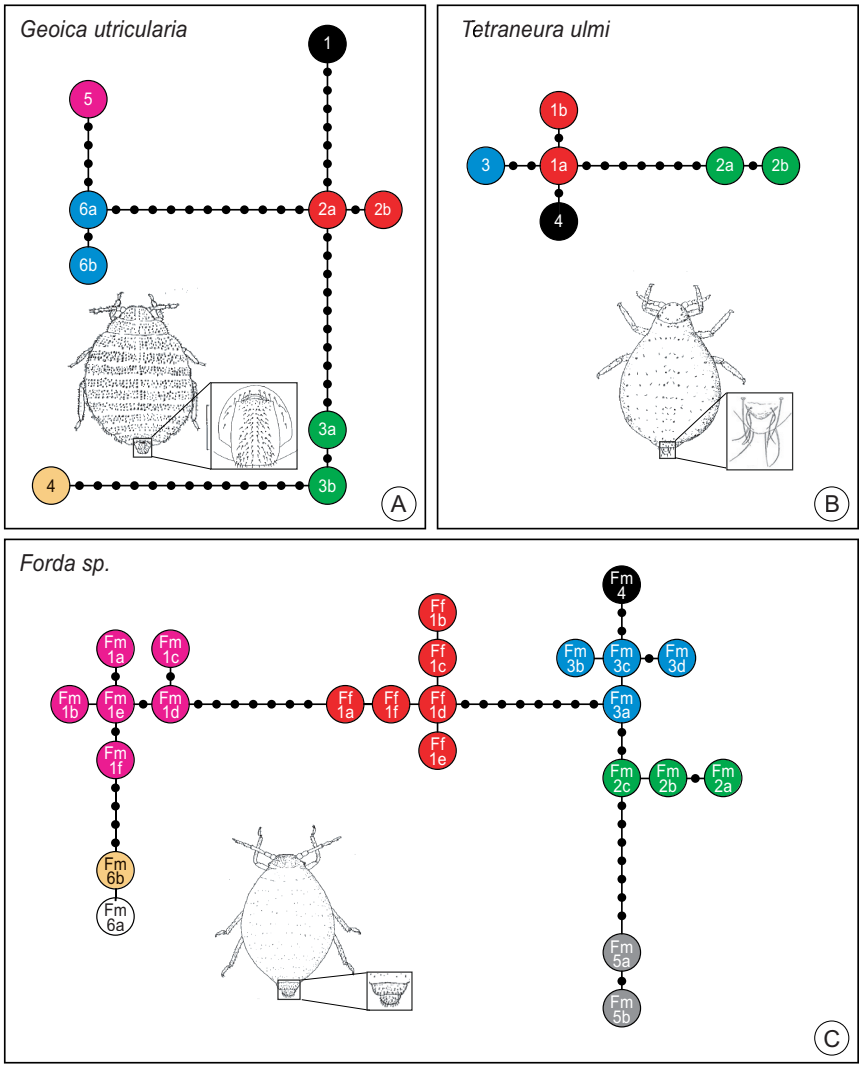


Figure 2.2 Typical habitus and minimum spanning MLG trees for the three root aphid genera. Drawings represent apterous viviparous females of *G. utricularia* (A), *T. ulmi* (B) and *F. marginata* (C). Adaptations to mymecophily, elongated anal hairs to hold honeydew, are highlighted (ventral view in A and B, dorsal view in C). All drawings are reproduced from Heie (1980). Multilocus genotype (MLG) trees were constructed based on shared allele distance (DAS), multiplied by the number of alleles available for comparison, to give the number of unshared alleles. Every circle represents a single MLG and names correspond to those used in Table 2.2, with colours representing multilocus lineages (MLLs). Connected MLGs differ from each other by a single allele and black dots represent potential unsampled haplotypes that differ from neighbours by a single allele. The *Forda* tree includes both *F. formicaria* (Ff- MLGs, all belonging to one MLL given in red) and *F. marginata* MLGs.

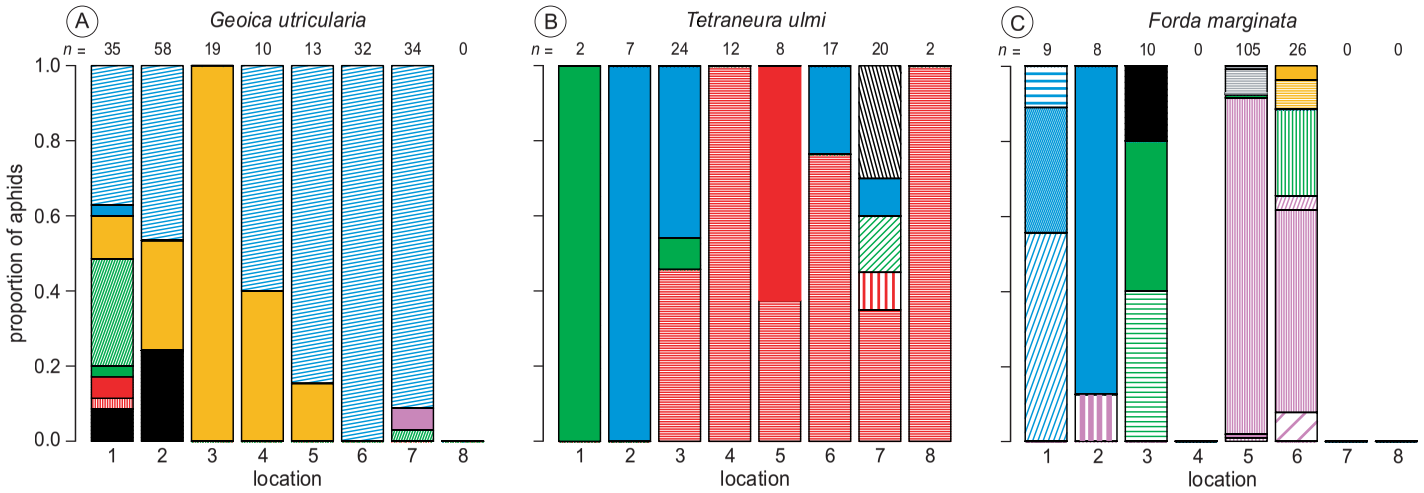


Figure 2.3 Spatial distribution of cumulative frequencies of multilocus lineages (MLLs) and multilocus genotypes (MLGs) for *Geoica utricularia* (A), *Tetraneura ulmi* (B) and *Forda marginata* (C). Hatching patterns within each column represent the MLGs, whereas colours represent the MLLs and correspond to the colours used in Figure 2.2. *n* is the sample size per aphid species for each transect location (location 1–8, with adjacent sample sites being 1 km apart – see Fig 2.1).

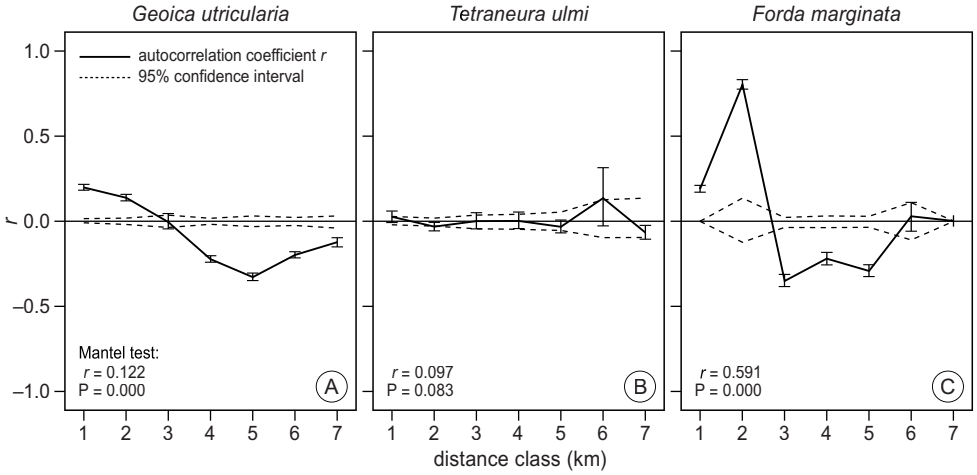


Figure 2.4 Spatial autocorrelation patterns for *Geoica utricularia* (A), *Tetraneura ulmi* (B) and *Forda marginata* (C). Plots show genetic correlation coefficients r (solid line), their 95% confidence bars as determined by bootstrapping, and the 95% confidence intervals around zero as expected from random distributions of genotypes (dashed lines), plotted across the seven distance classes (1–7 km). Results for the Mantel tests for each correlation are provided in each panel.

in this species. For completeness, the results of this analysis are given in the appendix (Figures 2.A2 and 2.A3).

We found alate aphids for all species except *F. formicaria*, but numbers were always very small (5 for *G. utricularia*, 1 for *T. ulmi*, and 4 for *F. marginata* out of a total of 505 aphids sampled in our entire study). Four of these alates were genotyped, which showed that three of them belonged to the same MLG as the other aphids sampled from the same ant nest, whereas the fourth alate had a different genotype (but one known from another colony nearby). Based on the observed alate frequencies, we estimated that these alate phenotypes had upper 95% CLs of 6% at most in the three species for which we had sample sizes to arrive at reasonable estimates (Table 2.3).

Table 2.3 Percentages of alates for the four root aphid species. Total number of collected aphids (*n*), the percentage of alates and the 95% confidence intervals around them (Rohlf & Sokal 1981).

Species	*n*	Observed alates (%)	Confidence intervals (%)
Geoica utricularia	225	2.2	0.6 – 4.8
Tetraneura ulmi	93	1.1	0.0 – 5.7
Forda marginata	169	2.4	0.5 – 5.4
Forda formicaria	18	0.00	0.0 – 15.1

DISCUSSION

Our population genetic estimates showed a strong signature of clonal reproduction for all four root aphid species. We therefore infer that *G. utricularia*, *T. ulmi*, *Forda marginata* and *F. formicaria* have predominantly if not exclusively asexual reproduction in the sampled Schiermonnikoog population, which largely confirms earlier findings and records (Muir 1959; Pontin 1978; Heie 1980; Godske 1991; Blackman & Eastop 1994). A single clone accounted for the majority of individuals in all species. In *G. utricularia* and *F. marginata*, genetic differentiation and geographical distance correlated significantly, further corroborating that the clonal aphid populations are viscous.

The reproductive modes of ant-tended root aphids

Anholocyclic reproduction and overwintering as asexual females (instead of sexually produced eggs) in ant nests in Northwest Europe has previously been inferred for *G. utricularia* and both *Forda* species (Muir 1959; Pontin 1978; Heie 1980; Godske 1991; Blackman & Eastop 1994), and our molecular data confirm this. However, finding the same extent of asexual reproduction in *T. ulmi* was interesting, as this result matched observations by (Muir 1959; Pontin 1978; Godske 1991), but contrasted with (Heie 1980) statement that *T. ulmi* reproduces sexually on elm trees in NW-Europe. Moderately mature (ca. 30 years old) elm trees do occur in the single village on the island of Schiermonnikoog, but leafgalls of *T. ulmi* were not found (A.B.F. Ivens & M. Schrama, unpublished data). The recurrence of identical *T. ulmi* clones in the same nest-mounds over consecutive years (Ivens *et al.* 2012a; Chapter 4) further corroborates that *T. ulmi* does not reproduce sexually on the island at any appreciable frequency. Until the 1980's there were much older elm trees in the village, which died from Dutch Elm Disease. These might have provided better opportunities for sexual reproduction in *T. ulmi*.

It is important to note that currently available statistical tests for inferring modes of reproduction from microsatellite data are designed to test the null hypothesis of 100% sexual reproduction and random mating, so that rejecting this null hypothesis does not imply that sexual reproduction is completely absent. Low frequencies of sex (<5%) could also account for the high variance in F_{IS} values in combination with the strong LD that we found in most of the loci-pairs (De Meeus & Balloux 2004). Statistically, we cannot rule out, therefore, that a small proportion of reproduction, particularly in *T. ulmi*, was in fact sexual (Balloux *et al.* 2003; Chapter 3), consistent with the primary host plant for this species being available and spatial autocorrelation being low, in contrast to *G. utricularia* and *F. marginata* where the host shrub for sexual reproduction is lacking (Heie 1980; Blackman & Eastop 1994) and autocorrelation patterns are distinct (Figure 4ab).

Population viscosity and dispersal

The aphid populations under study were viscous with low frequencies of alates and significant autocorrelation in two species, *G. utricularia* and *F. marginata*. These autocorrelations were tenfold stronger that the largely non-significant, previously observed autocorrelations in two species of holocyclic aphids (Abbot & Chhatre 2007; Michel *et al.* 2009). Despite this strong spatial structuring, some clonal lineages had managed to spread

along the studied transect (Figure 2.3), most likely by wind-dispersal of winged individuals (alates). As our genetic data indicate that there is negligible recruitment from sexual reproduction on Schiermonnikoog, this must imply that the few alates that we genotyped were either sexual migrants destined to fail (*i.e.* to never result in spring offspring recolonizing ant mounds), or unusual asexual dispersers headed for other ant mounds rather than for primary host trees. Both scenarios would be consistent with the alate MLGs being identical with those of wingless aphids in the same or a neighbouring mound, so that our data do not allow us to discriminate between these possibilities.

Morphological analysis of the embryos borne by the alates could have allowed us to resolve whether alates were sexual or not, since embryos of sexuparae are arostrate, whereas embryos of asexual dispersers are rostrate (Blackman & Eastop 1994). Unfortunately, by the time we realized this, we had used the alates for DNA extraction and microscopic preparation. However, the timing of their appearance (mid-summer) suggests that we were dealing with unusual asexual dispersers, as sexupara are expected to occur only towards the autumn (Heie 1980). Moreover, as the primary host of three species (the Mediterranean shrub *Pistacia*) has not occurred even close to the Island of Schiermonnikoog since the last glaciation, it seems hard to imagine how the island could have ever been colonized if alates of these species could only migrate to and from *Pistacia* shrubs.

What we can infer is that clonal aphid gene flow between ant mounds would indeed be very low if dispersal remains restricted to wingless aphids walking on the soil surface (even when helped somewhat by wind), which has been observed for both *Forda* and *Tetraneura* (Muir 1959) or passive floating during tidal inundations, which has been observed for the salt marsh root aphid *Pemphigus trehernei*, a non-ant-attended relative of *Forda* and *Geoica* (Foster & Treherne 1978; Foster 1978). *P. trehernei* also reproduces predominantly clonally and produces alates at very low frequencies (Foster 1975), but nevertheless colonizes new host plants very effectively (Foster & Treherne 1978). Although dispersal by tidal floating is poorly understood, it is conceivable that the aphid symbionts of *L. flavus* employ the same mechanism during occasional summer inundations, to colonize existing ant mounds where they might then establish a new MLG that can be vertically propagated after adoption by the ants (Ivens *et al.* 2012a; Chapter 4).

Why aphids may lose sex altogether and does ant-tending matter?

Although it has long been known that aphids either have holocyclic (with sex) or anholocyclic (completely parthenogenetic) life cycles, we lack an overall understanding of the selection forces that make aphids lose sex and whether such development is always irreversible. This is because populations of only a few species have been studied in sufficient genetic detail and few of these studies have targeted metapopulations consisting of holocyclic and anholocyclic patches. Polymorphisms of this kind are known to occur (Simon *et al.* 2002) and genetic studies of such populations would thus be highly informative. The strong LD, significant deviations from HWE and the observed heterozygote excess match the genetic population structures of other anholocyclic aphids (Simon *et al.* 1996; Simon *et al.* 1999; Delmotte *et al.* 2002; Gilabert *et al.* 2009; Kanbe & Akimoto 2009). However, all of these studies concern free-living aphids, while an association with ants is likely to affect

the genetic population structure as well. Yao (2010) for example showed that F_{IS} values for ant-tended *Tuberculatus* aphids were higher than those for unattended aphids. However, Vantaux *et al.* (2011a) did not find any effect of ant attendance on the genetic population structure of facultatively ant-tended aphids above ground. The root aphids studied here may have such metapopulations consisting of sexual and asexual patches in Southern Europe (see below), but their genetic analysis would only reveal whether close proximity of primary host plants increases the frequency of sex, and not whether ant tending matters, because there are no free living underground populations of these aphids (see Figure 2.2 for illustrations of their adaptations to myrmecophily).

Living in obligate mutualistic symbiosis has been argued to promote the irreversible loss of sexual reproduction when the symbiotic environment becomes highly predictable and uniform (Law & Lewis 1983). Although originally developed for endosymbionts, this hypothesis might also apply to ectosymbionts such as the aphids of our present study, because they are surrounded by a highly protective host colony (*i.e.* they are endosymbionts at the colony-level). The ants thus provide a protective underground environment in which potentially (1) sexually produced frost-resistant eggs are no longer necessary and (2) well-known sex-inducing environmental cues such as lower temperature and shorter daylight are less likely to be effective as cues (Rispe *et al.* 1998, reviewed in Moran 1992; Hales *et al.* 1997; Simon *et al.* 2002). In addition, ants actively keep aphid densities low by culling (Ivens *et al.* 2012a; Chapter 4), decreasing the potential for crowding, a factor also known to induce dispersal and sexual reproduction in aphids (reviewed in Hales *et al.* 1997). Dispersal constraints due to lack of primary host plants nearby and year-round underground nursing may thus have tipped the balance towards permanent parthenogenesis, relative to aphids that are facultatively ant-tended on food plants above ground where holocyclic life-cycles are more often maintained. We would thus be surprised if underground ant tending would ever be compatible with substantial aphid sexuality, even when primary host plants grow nearby. This logic seems to match the sparsely available comparative data as many above-ground aphids such as *Aphis fabae* combine a holocyclic life cycle with facultative ant-association (Heie 1986), whereas some other obligately ant-tended aphids and coccids have become anholocyclic and even use the dispersing virgin queens of the host ants for transmission (Hölldobler & Wilson 1990).

It is interesting to note that some studies have found that 'host-ant management' can delay aphid dispersal and reduce alate production (Way 1963; Kindlmann *et al.* 2007; Oliver *et al.* 2007; Yao 2010; Tegelaar *et al.* 2011). In addition to the culling mentioned above, underlying mechanisms for such practices as described for *Lasius* ants vary from inhibition through semiochemicals that slow aphids down (Oliver *et al.* 2007) to ants actively cutting the wings of their 'dairy farm' dispersal morphs (Way 1963; Hölldobler & Wilson 1990). We note, however, that our study population does not allow a convincing test of the possible role of 'ant management' in the restriction of root aphid sex, because the primary host plant was absent for three species and quite possibly unsuitable for the fourth species (see above). Such a study would be possible in the southern European range of *Pistacia*, at sites where these bushes and mature elm trees co-occur with *L. flavus* and their root aphids. The microsatellite markers developed for the present study would provide efficient tools to address these questions.

Acknowledgments

We thank Sylvia Mathiasen for help in the laboratory, Thijs Janzen for field and laboratory assistance, Ole E. Heie and Maurice Jansen for help with aphid taxonomy, David Nash for constructing the minimal spanning trees of Figure 2.2 and Subject Editor at *Molecular Ecology* Jacob Russell and three anonymous reviewers for their constructive comments on the manuscript. This study was supported by a PhD-Grant of the Centre for Ecological and Evolutionary Studies, University of Groningen and grants by the Langerhuizen Fund and Nicolaas Mulerius Fund, awarded to A.B.F.I, as well as by a grant from the Danish National Research Foundation to J.J.B that funded D.J.C.K's contributions before he obtained support from the Harvard Society of Fellows. We are grateful to the Vereniging Natuurmonumenten for granting us permission to conduct field work in the National Nature Reserve Schiermonnikoog and to the publishers Apollo Books, Stenstrup, Denmark and Brill, Leiden, the Netherlands for granting permission to reproduce figures from Heie (1980) in Figure 2.2.

Author information

This work is part of the PhD thesis of ABFI. All authors have long standing interest in the evolutionary biology of cooperation and conflict within and between species in all its facets, including reproductive mode and genetic diversity. FJW and IP are theoretical biologists using a modeling approach to study social evolution and the causes and consequences of sexual reproduction.

Data Accessibility

Sampling locations, sampling details, and microsatellite data are available from DRYAD (entry doi:10.5061/dryad.d0t63).

APPENDIX

The dependence of the ant *Lasius flavus* on root aphids

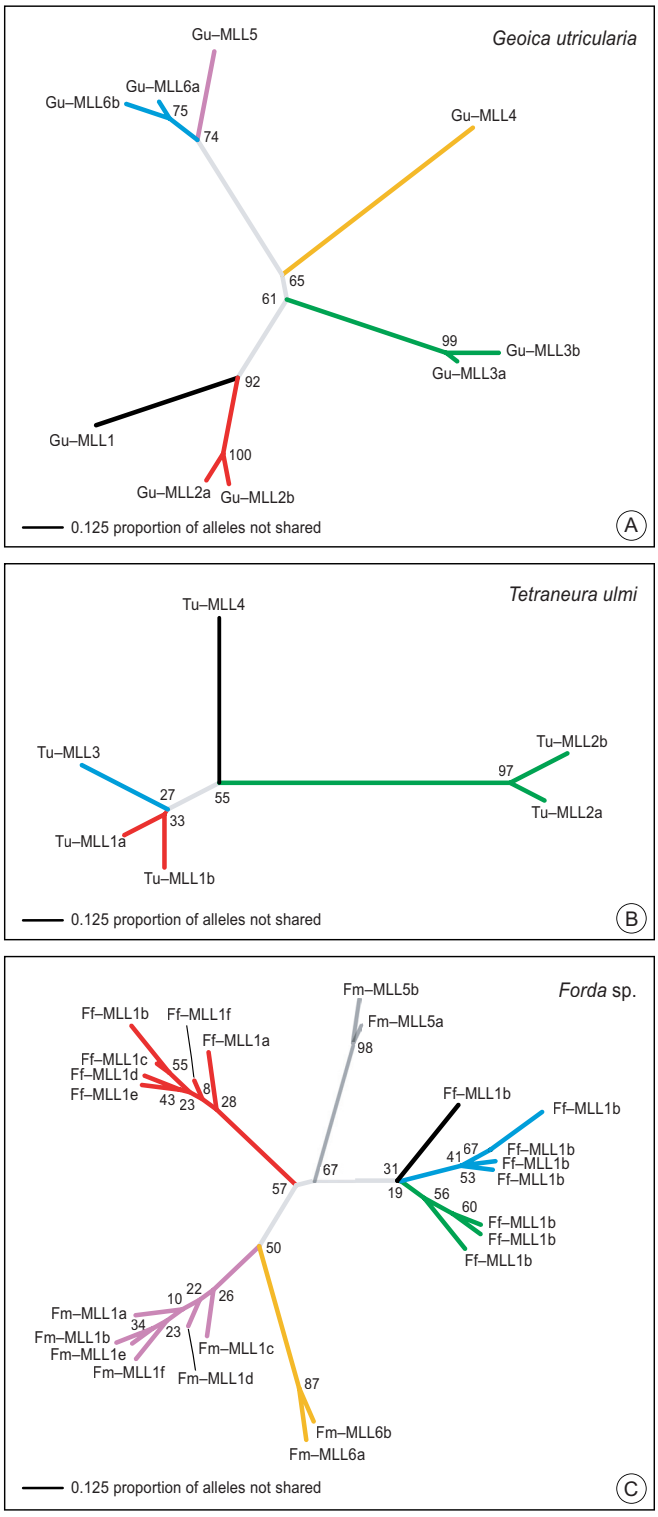
The honeydew secreted by the aphids is the main source of carbohydrates for the subterranean *L. flavus* ants, as other subterranean carbon sources are limited in the salt marsh and chalk-grassland habitats of these ants. Stable isotope analyses have confirmed that in the presence of aphids there is a demonstrable intermediate trophic level between the vegetation and the ants, consistent with the aphids being an intermediate foodweb node (A.B.F. Ivens & M. Schrama, unpublished data). *L. flavus* ants have also been recorded to eat a large proportion of their root aphids, suggesting that they also utilize their 'dairy cattle' to increase protein acquisition (Pontin 1958; 1961b; 1978; Ivens *et al.* 2012a; Chapter 4). This implies that, similar to other subterranean ants, *L. flavus* depends on root aphid husbandry for producing colonies large enough to reproduce (Way 1963; Hölldobler & Wilson 1990).

Table 2.A1 Results of tests for the presence of Linkage Disequilibrium for *ramet* and *genet* datasets of *Geoica utricularia*, *Tetraneura ulmi* and *Forda marginata*. G-tests for each available pair of loci were run using the Markov Chain algorithm of Raymond and Rousset (1995). P-values statistically significant at a 5%-level are given in bold, indicating pairs of loci with significant Linkage Disequilibrium. The parameter setting for dememorisation was 10000, the number of batches was set at 20, and the number of iterations per batch was 5000.

Ramet data				*Genet data*			
Locus 1	**Locus 2**	**P-Value**	**S.E.**	**Locus 1**	**Locus 2**	**P-Value**	**S.E.**
Geoica utricularia							
Gu2	Gu2	**0.000**	0.000	Gu2	Gu3	**0.000**	0.000
Gu2	Gu2	**0.000**	0.000	Gu2	Gu5	**0.001**	0.001
Gu2	Gu2	**0.000**	0.000	Gu2	Gu6	**0.016**	0.003
Gu2	Gu2	**0.000**	0.000	Gu2	Gu8	**0.008**	0.002
Gu2	Gu2	**0.000**	0.000	Gu2	Gu9	0.483	0.011
Gu2	Gu2	**0.000**	0.000	Gu2	Gu11	0.078	0.005
Gu2	Gu2	**0.000**	0.000	Gu2	Gu13	0.054	0.005
Gu5	Gu5	**0.000**	0.000	Gu5	Gu3	**0.001**	0.000
Gu5	Gu5	**0.000**	0.000	Gu5	Gu6	**0.003**	0.001
Gu5	Gu5	**0.000**	0.000	Gu5	Gu8	**0.003**	0.001
Gu5	Gu5	**0.000**	0.000	Gu5	Gu9	0.904	0.006
Gu5	Gu5	**0.000**	0.000	Gu5	Gu11	**0.010**	0.003
Gu5	Gu5	**0.000**	0.000	Gu5	Gu13	**0.011**	0.003
Gu6	Gu6	**0.000**	0.000	Gu6	Gu3	**0.003**	0.002
Gu6	Gu6	**0.000**	0.000	Gu6	Gu8	**0.007**	0.002
Gu6	Gu6	**0.000**	0.000	Gu6	Gu9	1.000	0.000
Gu6	Gu6	**0.000**	0.000	Gu6	Gu11	0.124	0.010
Gu6	Gu6	**0.000**	0.000	Gu6	Gu13	0.057	0.007
Gu8	Gu8	**0.000**	0.000	Gu8	Gu3	**0.002**	0.001
Gu8	Gu8	**0.000**	0.000	Gu8	Gu9	0.235	0.010
Gu8	Gu8	**0.000**	0.000	Gu8	Gu13	**0.002**	0.001
Gu9	Gu9	**0.000**	0.000	Gu9	Gu3	0.903	0.007
Gu9	Gu9	**0.000**	0.000	Gu9	Gu13	0.097	0.009
Gu11	Gu11	**0.000**	0.000	Gu11	Gu3	**0.012**	0.003
Gu11	Gu11	**0.000**	0.000	Gu11	Gu8	**0.049**	0.004
Gu11	Gu11	**0.000**	0.000	Gu11	Gu9	0.338	0.013
Gu11	Gu11	**0.000**	0.000	Gu11	Gu13	**0.018**	0.003
Gu13	Gu13	**0.000**	0.000	Gu13	Gu3	**0.009**	0.002
Pairs with significant LD : 28				**Pairs with significant LD : 17**			

Table 2.A1 Continued

Ramet data				*Genet data*			
Locus 1	**Locus 2**	**P-Value**	**S.E.**	**Locus 1**	**Locus 2**	**P-Value**	**S.E.**
Tetraneura ulmi							
Tu1	Tu3	**0.000**	0.000	Tu1	Tu3	0.467	0.004
Tu1	Tu11	0.073	0.003	Tu1	Tu11	1.000	0.000
Tu2	Tu1	**0.000**	0.000	Tu2	Tu1	0.066	0.002
Tu2	Tu3	**0.000**	0.000	Tu2	Tu3	0.465	0.006
Tu2	Tu4	**0.000**	0.000	Tu2	Tu4	0.205	0.003
Tu2	Tu11	0.070	0.002	Tu2	Tu11	1.000	0.000
Tu4	Tu1	**0.000**	0.000	Tu4	Tu1	0.198	0.003
Tu4	Tu3	**0.000**	0.000	Tu4	Tu3	1.000	0.000
Tu4	Tu11	**0.000**	0.000	Tu4	Tu11	1.000	0.000
Tu10	Tu1	0.095	0.002	Tu10	Tu1	1.000	0.000
Tu10	Tu2	0.091	0.002	Tu10	Tu2	1.000	0.000
Tu10	Tu3	**0.000**	0.000	Tu10	Tu3	0.399	0.006
Tu10	Tu4	**0.000**	0.000	Tu10	Tu4	1.000	0.000
Tu10	Tu11	**0.000**	0.000	Tu10	Tu11	0.194	0.005
Tu11	Tu3	**0.000**	0.000	Tu11	Tu3	0.208	0.011
Pairs with significant LD : 11				**Pairs with significant LD : 0**			
Forda marginata							
Fm1	Fm3	**0.000**	0.000	Fm1	Fm3	**0.001**	0.001
Fm1	Fm4	**0.000**	0.000	Fm1	Fm4	**0.000**	0.000
Fm1	Fm6	**0.000**	0.000	Fm1	Fm6	**0.001**	0.001
Fm1	Gu6	**0.000**	0.000	Fm1	Gu6	0.870	0.006
Fm1	Gu11	**0.000**	0.000	Fm1	Gu11	**0.000**	0.000
Fm1	Gu13	**0.000**	0.000	Fm1	Gu13	**0.033**	0.006
Fm3	Fm4	**0.000**	0.000	Fm3	Fm4	**0.000**	0.000
Fm3	Fm6	**0.000**	0.000	Fm3	Fm6	**0.000**	0.000
Fm3	Gu6	**0.000**	0.000	Fm3	Gu6	0.060	0.005
Fm3	Gu11	**0.000**	0.000	Fm3	Gu11	**0.000**	0.000
Fm3	Gu13	**0.000**	0.000	Fm3	Gu13	**0.003**	0.001
Fm4	Fm6	**0.000**	0.000	Fm4	Fm6	**0.000**	0.000
Fm4	Gu6	**0.000**	0.000	Fm4	Gu6	0.537	0.009
Fm4	Gu11	**0.000**	0.000	Fm4	Gu11	**0.000**	0.000
Fm4	Gu13	**0.000**	0.000	Fm4	Gu13	**0.001**	0.001
Fm6	Gu6	**0.000**	0.000	Fm6	Gu6	0.167	0.006
Fm6	Gu11	**0.000**	0.000	Fm6	Gu11	**0.000**	0.000
Fm6	Gu13	**0.000**	0.000	Fm6	Gu13	**0.000**	0.000
Gu6	Gu11	**0.000**	0.000	Gu6	Gu11	**0.023**	0.003
Gu13	Gu6	**0.000**	0.000	Gu13	Gu6	0.107	0.004
Gu13	Gu11	**0.000**	0.000	Gu13	Gu11	**0.001**	0.000
Pairs with significant LD : 23				**Pairs with significant LD : 16**			


Geoica utricularia
Gu–MLL5
Gu–MLL6a
Gu–MLL6b
75
74
Gu–MLL4
65
61
99
Gu–MLL3b
Gu–MLL3a
92
Gu–MLL1
100
Gu–MLL2a
Gu–MLL2b
0.125 proportion of alleles not shared
A
Tetraneura ulmi
Tu–MLL4
Tu–MLL3
27
55
Tu–MLL2b
97
Tu–MLL2a
33
Tu–MLL1a
Tu–MLL1b
0.125 proportion of alleles not shared
B
Forda sp.
Fm–MLL5b
Fm–MLL5a
98
Ff–MLL1b
Ff–MLL1f
Ff–MLL1a
Ff–MLL1c
55
Ff–MLL1d
Ff–MLL1e
43
23
8
28
Ff–MLL1b
Ff–MLL1b
67
41
Ff–MLL1b
Ff–MLL1b
53
Ff–MLL1b
67
31
57
19
56
60
Ff–MLL1b
Ff–MLL1b
Ff–MLL1b
50
10
22
26
Fm–MLL1a
34
23
Fm–MLL1b
Fm–MLL1e
Fm–MLL1f
Fm–MLL1c
Fm–MLL1d
87
Fm–MLL6b
Fm–MLL6a
0.125 proportion of alleles not shared
C

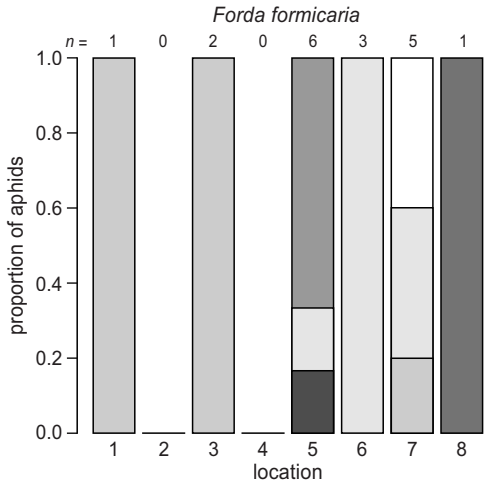


Figure 2.A2 Spatial distribution of cumulative frequencies of Multilocus Genotypes (MLGs) of *Forda formicaria*. Distinct MLGs are represented by different hatching patterns. *n* gives the sample size for each transect location (location 1-8, with adjacent sample sites being 1 km apart – see Fig 2.1).

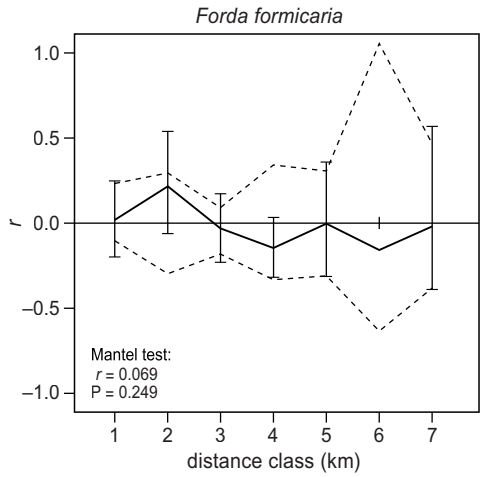


Figure 2.A3 Spatial autocorrelation pattern for *Forda formicaria*. The plot shows genetic correlation coefficient *r* (solid line), its 95% confidence bars as determined by bootstrapping, and the 95% confidence interval around zero as expected from random distributions of genotypes (dashed lines), plotted across the seven distance classes (1–7 km). The result for the Mantel test of the correlation is provided in the left corner of the panel. Due to small sample sizes (see Figure 2.A2), these tests remain inconclusive.

Figure 2.A1 (left) Neighbour-Joining trees for the three root aphid genera. Neighbour-Joining trees were constructed based on shared allele distances (DAS) (Jin & Chakraborty 1993). Genetic distances were calculated and trees were constructed in POPULATIONS 1.2.30 (Olivier Langella 1999) and displayed graphically in R 2.12.0 (R development core team 2010) using the plot.phylo() function in the ape package (Paradis *et al.* 2004). Genetic distance in the trees is given as the proportion of alleles that are not shared between multilocus genotypes (MLGs). Colours represent specific multilocus lineages (MLLs) consisting of several multilocus genotypes (MLGs) with names corresponding to those given in Table 2.2 and node numbers representing bootstrap values (10000 iterations). The *Forda* tree includes both *F. formicaria* (Ff- MLGs, all belonging to one MLL given in red) and *F. marginata* MLGs.

CHAPTER 3

Estimating the frequency of sexual reproduction in root aphids based on microsatellite markers

Aniek B.F. Ivens
Jacobus J. Boomsma
Franz J. Weissing
Ido Pen

ABSTRACT

Many organisms are known to have a mixed reproductive strategy of both sexual and asexual reproduction. Mixed reproduction can have major implications for the ecology and evolution of a species and detecting the occurrence of mixed reproduction and its extent can therefore be crucial for our understanding of the evolutionary ecology of these organisms. However, to date it remains difficult to indirectly estimate the frequency of sexual and asexual reproduction from microsatellite data. This is because traditional methods remain limited to all-or-nothing detection of sexual reproduction, rather than allowing for estimation of rates sexual reproduction smaller than 100%. Here, we present a simulation model of population genetic diversity under varying rates of sexual reproduction. Population genetic diversity is assessed using two estimates: clonal diversity P_d and effective clonal diversity P_{de}. Model assumptions of microsatellite marker diversity and spatial population structure were especially tailored to root aphids and based on empirical data of natural population of these cyclic parthenogens. The results show that in predominantly sexual populations, small amounts of asexual reproduction will remain hard to detect. On the other hand, small amounts of sexual reproduction in predominantly clonal populations leave significant population genetic signatures. The results further show that the clonal diversity observed for the root aphids on which we based our simulations, match best with predominant asexual reproduction and absence of dispersal. For one root aphid species, *Tetraneura ulmi*, however, observed genotypic variation proved too small to draw definite conclusions from our simulations and neither did our simulation results match earlier findings with traditional methods. We therefore argue that for inferring mixed reproduction from microsatellite data a pluralistic approach consisting of several different analyses should ideally be applied.

INTRODUCTION

A small amount of sexual reproduction or recombination can overcome the costs of strictly asexual reproduction, which is generally considered an evolutionary 'dead end' (Green & Noakes 1995; Hurst & Peck 1996; D'Souza & Michiels 2010). These costs, such as accumulation of deleterious mutations ('Muller's ratchet') and being 'out-evolved' by parasites may ultimately outweigh the benefits of the higher reproductive rate of clonal species (not having to find a mate) and the preservation of beneficial combinations of alleles in absence of recombination (reviewed in Green & Noakes 1995). Detecting a small proportion of sexual reproduction in an otherwise clonal organism can thus be crucial for our understanding of the ecology and evolution of the species under study. *Vice versa* can small bouts of asexual reproduction in a predominantly sexual species allow for increased colonization opportunities, through outbursts of high reproductive rates. Many of the species with potentially mixed reproductive modes include ecologically and economically important species such as various insects that are crop pests (*e.g.* aphids), crop plants (*e.g.* potato) and human pathogens (*e.g. Candida albicans*) (reviewed in De Meeus *et al.* 2007). Moreover, the frequency of sexual reproduction can have a strong effect on effective population sizes (N_E) (Yonezawa *et al.* 2004), which can have important consequences for the conservation genetics of a species. However, despite its assumed importance, it was not until recently that attention shifted towards the study of mixed reproduction in single, natural populations (among others Balloux *et al.* 2003; De Meeus *et al.* 2006; Villate *et al.* 2010; Allen & Lynch 2012).

A major reason for our lack of knowledge on the occurrence of mixed reproductive modes in natural populations is the lack of accurate methods to estimate the frequency of sexual reproduction from population genetic data. The most widely used techniques are tests for deviations from Hardy-Weinberg Equilibrium (HWE) and estimating so-called P_{sex} values for specific genotypes. The P_{sex} value of a genotype gives the probability that the number of repeated observations of a particular genotype in the population resulted from independent events of sexual reproduction, rather than asexual reproduction (Tibayrenc *et al.* 1990; Parks & Werth 1993; Young *et al.* 2002; Arnaud-Haond *et al.* 2007). Both methods rely on rejecting the null hypothesis of 100% random mating, with the only available alternative being 100% clonal reproduction and thus leaving lower rates of sexual reproduction undetected (reviewed in Arnaud-Haond *et al.* 2007). Moreover, both HWE and P_{sex} approaches test observed population genetic data under the classic, restricted, assumptions of a randomly mating, unstructured population. In sum, the currently often observed 'negative' result of failing to detect sexual reproduction with these classic approaches could also be explained by (1) proportions of sexual reproduction much smaller than 100% occurring in a population, which remain undetected by current methods and (2) the 'ideal' HWE assumptions not being met; violation of any of these assumptions will cause significant deviations from HWE.

Several theoretical studies have investigated the effects of mixed reproductive strategies on the patterning of genetic variation in a population (Green & Noakes 1995; Balloux *et al.* 2003; Bengtsson 2003; De Meeus & Balloux 2004; Halkett *et al.* 2005; D'Souza & Michiels 2010). These studies reveal that proportions of clonality other than those

approaching 100% remain hard to detect from population genetic data alone (Balloux *et al.* 2003; De Meeus & Balloux 2004; De Meeus *et al.* 2006), despite the fact that a combination of sexual and asexual reproduction is known to have large effects on allelic divergence, effective population sizes and genotypic diversity of populations (Balloux *et al.* 2003; De Meeus & Balloux 2004; Halkett *et al.* 2005).

Only few empirical studies of species with mixed reproduction are available (reviewed in Halkett *et al.* 2005). An important example is the recent study by Allen & Lynch (2012), which used microsatellites to estimate genetic population structures of *Daphnia pulicaria* in six lakes known to differ in the relative frequency of sexual reproduction. D'Souza and Michiels (2006) provided similar data on freshwater planarian *Schmidtea ploychro*. Both studies show results that are in line with earlier theoretical predictions: populations with lower frequencies of sex show departures from HWE, have lower genotypic diversity and exhibit increased occurrence of linkage disequilibrium (LD) (D'Souza & Michiels 2006; Allen & Lynch 2012). These results can be explained by reduced recombination rates (Balloux *et al.* 2003; De Meeus & Balloux 2004; Halkett *et al.* 2005; Arnaud-Haond *et al.* 2007). In addition, in their 2010 study Villate *et al.* (2010) developed methods to identify sexual individuals in a predominantly clonal population. These methods combine the insights gained from the above discussed theoretical studies with classic HWE methods, polymorphic microsatellite markers, hierarchical sampling and phylogenetic reconstructions (Villate *et al.* 2010).

With only few empirical studies addressing population genetic structures under mixed reproduction, theory and data of mixed reproduction remain poorly linked. The theoretical models available might not apply directly to natural populations, because the underlying assumptions of high initial variation (*e.g.* 20 loci with each 99 alleles in Balloux *et al.* 2003) and spatial structure may not be biologically realistic for the species under empirical study. Here, we consider a model that closely resembles the individual-based simulation model of Balloux *et al.* (2003) and De Meeus & Balloux (2004), but that has been especially tailored to the biology of an example organism with mixed reproduction, the root aphid. Root aphid reproduction is similar to other aphids that are cyclical parthenogens, which alternate sexual and asexual reproduction (Simon *et al.* 2002). The root aphid species studied here are well-known for their mutualistic interaction with ants (Paul 1977; Pontin 1978; Heie 1980; Godske 1991; Ivens *et al.* 2012a; 2012b; Chapter 2; 4). Evolutionary theory predicts that their myrmecophilous lifestyle may select for reduced frequency of sexual reproduction (Wulff 1985; Chapter 2). The number of loci and associated alleles assumed in our model are based on microsatellite marker data of natural populations of three species of root aphids (Ivens *et al.* 2012a; 2012b; Chapter 2; 4). The spatial population structure in our simulations was modeled linear after the assumed between-patch dispersal tendencies of root aphids and in accordance with the transect-sampling in empirical studies (Ivens *et al.* 2012a; 2012b; Chapter 2; 4). The use of empirically collected data allows us to compare simulation outcomes on genotypic variation to those observed in the natural populations of our study species. This comparison provided a further check of the outcomes of theoretical models on the population genetics of mixed reproduction, especially for cases in which standard HWE assumptions are not met (Bengtsson 2003).

MODEL

In our simulations, we considered a monoecious population consisting of a linear array of 10 patches that each contain 1000 diploid individuals, in correspondence with the island model used by Balloux *et al.* (2003). The simulation assumes that these individuals reproduce sexually with fixed probability P_s and asexually with the complementary probability $1–P_s$.

During sexual reproduction, recombination occurs between two randomly drawn parents, whereas offspring produced by asexual reproduction are identical copies of their parent. The population was further assumed to be monoecious without selfing (unlike the model by Balloux *et al.* (2003)). The number of loci, allelic variation and initial allele frequencies were identical to those observed in the field for three species of root aphids (see below, Table 3.A1). During the reproduction phase, alleles mutate with probability $\mu = 10^{-3}$ into one of the other available alleles for that locus. By choosing this mutation implementation we followed Balloux *et al.* (2003), although microsatellite loci are likely to be subject to stepwise mutation (Nauta & Weissing 1996). The assumed mutation rate is higher than the rate of 10^{-5} applied by Balloux *et al.* (2003). This is because the naturally observed variation on which we based our simulations is lower than the theoretical variation assumed in Balloux *et al.* (2003) and De Meeus & Balloux (2004); under a low mutation rate of 10^{-5} drift would therefore erode all variation over simulation time. In addition, we assume that all loci are neutral and unlinked. After reproduction, parents die and the offspring disperse between patches with fixed probability P_{disp} and across a distance randomly chosen between 0 and a fixed maximal dispersal distance D_{dist}. After dispersal, 1000 juveniles per patch mature into the next parent generation. We assumed no fitness differences between sexually and asexually produced individuals and simulated 15000 generations, after which observed diversity estimates (see below) were in equilibrium (Figures 3.1, 3.A1).

Two indicators for population genetic diversity were estimated: clonal diversity P_d and effective clonal diversity P_{de}. The clonal diversity index P_d is given by G/N with G being the number of unique multilocus genotypes (MLGs) and N the total population size (Ellstrand & Roose 1987; Dorken & Eckert 2001; Arnaud-Haond *et al.* 2007). P_d is a widely used estimate of genotypic diversity in clonal species (reviewed in Arnaud-Haond *et al.* 2007). P_d ranges between 0 and 1, with a value of 1 indicating high genetic diversity (i.e every individual having a unique genotype). Population structures of clonal species are often characterized by one or a few MLGs that are overrepresented. To quantify this effect, we used the effective number of genotypes G_e (Balloux *et al.* 2003) given by:

$$G_e = \frac{1}{\sum g_i^2} \tag{2.1}$$

in which g_i is the frequency of the ith genotype. To normalize the effective number of genotypes for population size we use the following modified estimate for effective clonal diversity P_{de}

$$P_{de} = \frac{G_e}{N} \tag{2.2}$$

in our further analysis. Again, P_{de} ranges between 0 and 1 and values approaching 1 indicate high genetic diversity.

We ran simulations with the frequency of sexual reproduction P_s increasing from 0 and 1, in steps of size 0.01. To study the effect of population structure, these simulations were run for a series of dispersal regimes (P_{disp} = 0.0, 0.001, 0.01, 0.05, 0.1 in combination with D_{disp} = 1 and D_{disp} = 2, 5, 10 with P_{disp} = 0.01). All simulations were replicated 5 times.

Simulations were run using a C^{++} routine (available upon request) and compiled in g^{++} 4.3.4 (Free Software Foundation, Inc. 2008). The program included a random number generator that uses the GNU Scientific Library (James 1994; Lüscher 1994).

Data

The number of loci and allelic variation and frequency implemented in the model were based on population genetic data on three species of root aphids: *Geoica utricularia*, *Forda marginata* and *Tetraneura ulmi* (Ivens *et al.* 2012a; 2012b; Chapter 2; 4). Reproductive mode in aphids is known to differ between species and between populations of the species, with holocyclic species or populations having a sexual phase and anholocyclic species completely lacking this phase (Simon *et al.* 2002). However, the occurrence of rare sex might remain undetected in aphids that are thought to be anholocyclic. All aphids were sampled on the Dutch island of Schiermonnikoog in July 2008. To determine MLGs in these three species species-specific 5–8 polymorphic microsatellite markers (Ivens *et al.* 2011; Chapter 5) were used and sample sizes per species ranged from 92 to 201 individuals. A detailed description of the sampling, molecular analysis and observed MLGs can be found in Chapter 2.

RESULTS AND DISCUSSION

We simulated populations with the number of loci and allelic diversity observed in natural populations of three species of root aphids. Only the estimated microsatellite variation of *Geoica utricularia* and *Forda marginata* turned out to be sufficient for any effect to be observed over simulation time in our model. We will therefore focus discussion of the results on these two species. For completeness, *Tetraneura ulmi* results are given in the appendix (Figures 3.A1, 3.A2)

The effects of sexual reproduction and dispersal on clonal diversity

P_d and P_{de} values both fluctuated strongly over simulation time, with the strongest fluctuations observed for P_{de} values and under P_s = 1 (complete sexual reproduction), as is shown in Figure 3.1 for one representative simulation run. After an initial decline in clonal diversity over the whole range of P_s values, the diversity estimates kept fluctuating within a limited range of values. Caution should thus be taken at what time step observed and simulated diversity estimates are compared. Figure 3.2 shows results for generation 15000, corresponding to a near-equilibrium for all P_s values except maybe for complete sexuality (P_s = 1.0).

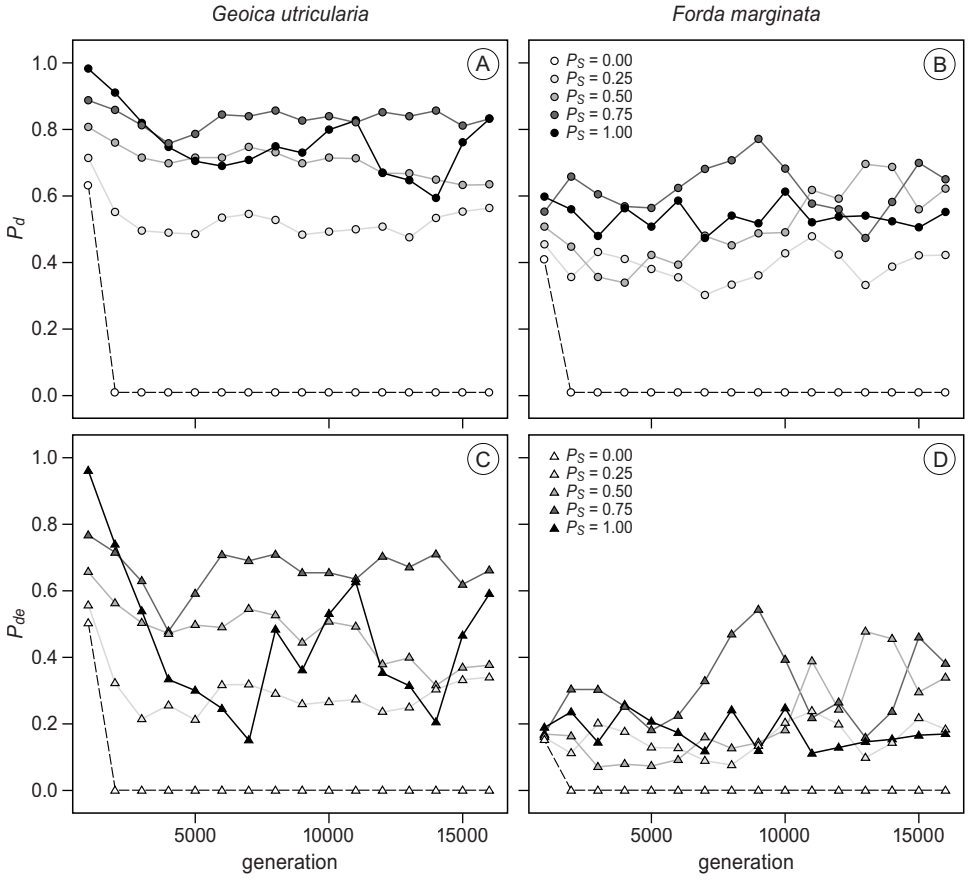


Figure 3.1 Clonal diversity P_d (top panels) and the effective clonal diversity P_{de} (bottom panels) over simulation time for varying frequencies of sexual reproduction. Simulations were run for two species of root aphids *Geoica utricularia* (A, C), *Forda marginata* (B, D). Plots give P_d and P_{de} for 15000 generations, for five frequencies of sexual reproduction P_s (see legends). In all simulations dispersal probability P_{disp} was 0.01 and maximal dispersal distance D_{dist} was 1.

Both measures of genetic diversity used in our study, clonal diversity P_d and effective clonal diversity P_{de} increased with increasing frequency of sexual reproduction, with the exception of the scenario without dispersal ($P_{\text{disp}} = 0.0$, Figure 3.2AB). This effect is in accordance with previous theoretical predictions by Balloux *et al.* (2003) and De Meeus & Balloux (2004), although the implemented, empirically estimated, locus and allelic variation were considerably lower than those simulated in the previous completely theoretical studies (6-8 loci with 4-9 alleles in our study compared to 20 loci with 99 alleles used in the previous studies, Table 3.A1). The curves for P_d and P_{de} level off towards P_s values of 100% sexual reproduction. This shows that it will be more difficult to detect small proportions of asexual reproduction in predominantly sexual populations than it will be to detect small bouts of sexual reproduction in a predominantly clonal population (see also D'Souza

& Michiels 2006; De Meeus *et al.* 2006). Indeed, a small percentage of sexual reproduction is known to have a major effect on patterning of genetic diversity in a population and can make a predominantly clonal population look like a sexually reproducing population. This is in line with conclusions that 'a little bit of sexual reproduction' may be superior to both purely clonal reproduction and purely sexual reproduction, since it combines the benefits of sexual reproduction (a high degree of genetic diversity) without having to bear the 'costs of sex' (Green & Noakes 1995; Bengtsson 2003; D'Souza & Michiels 2010).

Higher dispersal rates P_{disp} have a clear positive effect on clonal diversity (Figure 3.2AB), while dispersal distance D_{dist} has only a marginal additional effect (Figure 3.2CD). The positive effect of dispersal on clonal diversity under varying frequencies of sex is in accordance with previous results by De Meeus & Balloux (2004).

Insights into root aphid dispersal and reproduction

Observed P_d and P_{de} values in the natural populations of *G. utricularia* and *F. marginata* were consistently lower than those obtained in the simulations, even when assuming complete absence of sex (indicated by the arrows on the y-axes, Figure 3.2), with the exception of simulated populations that were both strictly asexual and highly structured ($P_{\text{disp}} = 0.0$). This shows that the observed clonal diversity for *G. utricularia* and *F. marginata* can be best explained by strong population structure in combination with exclusive asexual reproduction. The simulations thus enable us to confirm our previous findings of HWE-based analyses (Ivens *et al.* 2012b; Chapter 2). Predominant asexual reproduction and rare dispersal are also in accordance with the biological details known for these aphid species (Heie 1980; Ivens *et al.* 2012b; Chapter 2).

The same HWE-based analyses (Ivens *et al.* 2012b; Chapter 2), however, also rejected sexual reproduction in the third population of aphid species *Tetraneura ulmi* (Figure 3.A2), whereas our simulation results show that based on its observed genotypic variation, sexual reproduction cannot be completely excluded, because observed and simulated P_d and P_{de} values overlapped along the full range of P_s values from 0 to 1. This shows that caution should be taken when rejecting sexual reproduction based on classical HWE-tests alone, especially when standard assumptions, such as an unstructured population, are not met. The difference between the results for this species and the other two species may be due to lower statistical power because variation at marker loci was less pronounced in *T. ulmi* (6 loci, 2–5 alleles; rather than 6–7 loci with 8–9 alleles in the other two species, Table 3.A1). Alternatively, the ambiguous results for *T. ulmi* may reflect that some sex in this species does indeed occur, or did so until a few decades ago, as *T. ulmi* has mature elm trees (*Ulmus*) as primary hosts, which did in fact occur on the island until the 1980s, whereas the *Pistacia* primary host of *G. utricularia* and *F. marginata* is restricted to Mediterranean areas (Heie 1980; Ivens *et al.* 2012b; Chapter 2).

Conclusion and future directions

Our present simulations were restricted to microsatellite variation of three species of root aphids, with similar natural history characteristics. Further understanding of the importance of mixed reproductive modes in structured populations, would greatly benefit from repeating our simulations with data from other taxa than root aphids. Such future studies

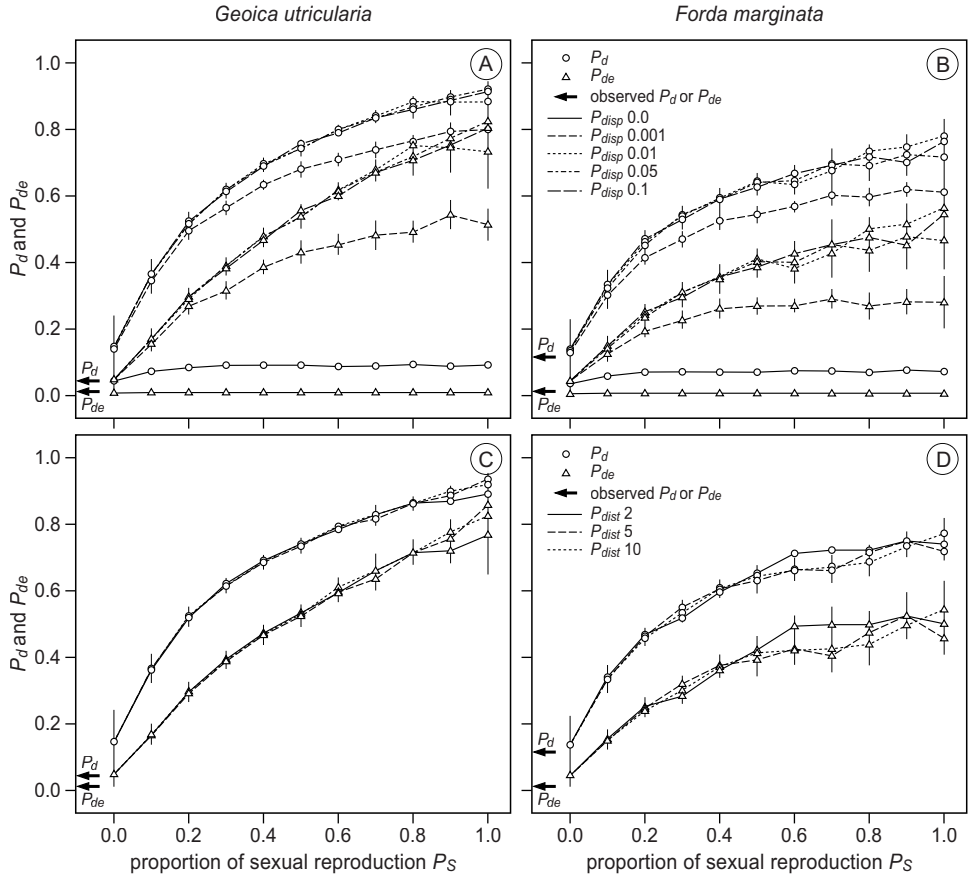


Figure 3.2 Clonal diversity P_d and effective clonal diversity P_{de} under increasing rate of sexual reproduction P_s for various dispersal scenarios. Simulations were run for two species of root aphids *Geoica utricularia* (A, C) and *Forda marginata* (B, D). Simulated P_d values for five dispersal probabilities and fixed dispersal distance (1) are given, and simulated P_{de} values for three dispersal distances and fixed dispersal probability (0.01). Error bars represent 95%-confidence intervals over five replicate simulations. Arrows indicate the observed estimates for P_d and P_{de} in the sampled populations of *G. utricularia* and *F. marginata*.

would be most informative if the frequency of sexual reproduction would be known from independent estimates based on other such as the presence and abundance of eggs (*e.g.* Allen & Lynch 2012). However, even these methods would only yield indirect estimates for the rate of sexual reproduction. Future laboratory studies on populations with controlled rates of sexual reproduction would be able to fill this gap and allow further validation of these models and their underlying assumptions on population genetic variation and population structure.

Our results show that detecting mixed reproductive modes, in particular small bouts of clonality, remains a challenge and that the complete absence of sex cannot be inferred

with certainty in cases where variation at marker loci is limited (*e.g. T. ulmi*). Ideally, conclusions on estimated rates of (a)sexual reproduction in natural populations with mixed reproductive strategies should therefore be based on multiple different analyses such as classic LD-, FIS- and P_{sex}-analyses combined with P_d simulations as presented here (Bengtsson 2003; De Meeus & Balloux 2004; Halkett *et al.* 2005; De Meeus *et al.* 2006; Arnaud-Haond *et al.* 2007).

Acknowledgements

We authors thank the Donald Smits Centre for Information Technology, Groningen, for letting us use the Millipede High Performance Cluster to run simulations. This study was supported by a PhD grant of the Centre for Ecological and Evolutionary Studies, University of Groningen and grants by the Langerhuizen Fund and the Nicolaas Mulerius Fund, awarded to A.B.F.I.

APPENDIX

Observed microsatellite diversity

In our simulations, the number of microsatellite loci, the number of alleles associated with these loci and the initial allele frequencies were identical to those observed in natural populations of the three root aphid species *Geoica utricularia*, *Forda marginata* and *Tetraneura ulmi*. Table 3.A1 gives the number of loci, number of associated alleles and allele frequencies used in our simulations of the three species.

Simulation results for root aphid *Tetraneura ulmi*

The genotypic variation observed in the natural populations of root aphids used in our simulations was considerably lower than the variation implemented in the models by Balloux *et al.* (2003) and De Meeus & Balloux (2004). Nevertheless, our simulations yielded very similar results for *G. utricularia* and *F. marginata*. However, the genotypic variation observed for *T. ulmi* was insufficient to offer decisive conclusions on the complete absence of sex in the simulations. Figures 3.A1 and 3.A2 give the simulation results for *T. ulmi.*

Table 3.A1 Allele frequencies used to initialize simulations for three species of root aphids.

Allele	Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6	Locus 7	Locus 8
Geoica utricularia								
Allele 1	0.042	0.057	0.005	0.060	0.100	0.109	0.100	0.042
Allele 2	0.117	0.612	0.010	0.010	0.612	0.114	0.030	0.042
Allele 3	0.114	0.030	0.085	0.308	0.259	0.055	0.114	0.007
Allele 4	0.612	0.229	0.114	0.042	0.030	0.114	0.114	0.037
Allele 5	0.114	0.030	0.114	0.308		0.299	0.306	0.306
Allele 6			0.612	0.271		0.005	0.301	0.450
Allele 7			0.030			0.299	0.030	0.110
Allele 8			0.030			0.005	0.005	
Forda marginata								
Allele 1	0.735	0.110	0.735	0.371	0.006	0.277	0.052	
Allele 2	0.029	0.006	0.026	0.158	0.258	0.358	0.106	
Allele 3	0.035	0.052	0.013	0.106	0.006	0.358	0.055	
Allele 4	0.013	0.103	0.116	0.365	0.729	0.003	0.052	
Allele 5	0.023	0.729	0.061			0.003	0.735	
Allele 6	0.065		0.048					
Allele 7	0.048							
Allele 8	0.026							
Allele 9	0.026							
Tetraneura ulmi								
Allele 1	0.837	0.924	0.429	0.924	0.766	0.332		
Allele 2	0.130	0.076	0.098	0.076	0.038	0.033		
Allele 3	0.033		0.022		0.163	0.076		
Allele 4			0.429		0.033	0.391		
Allele 5			0.022			0.168		

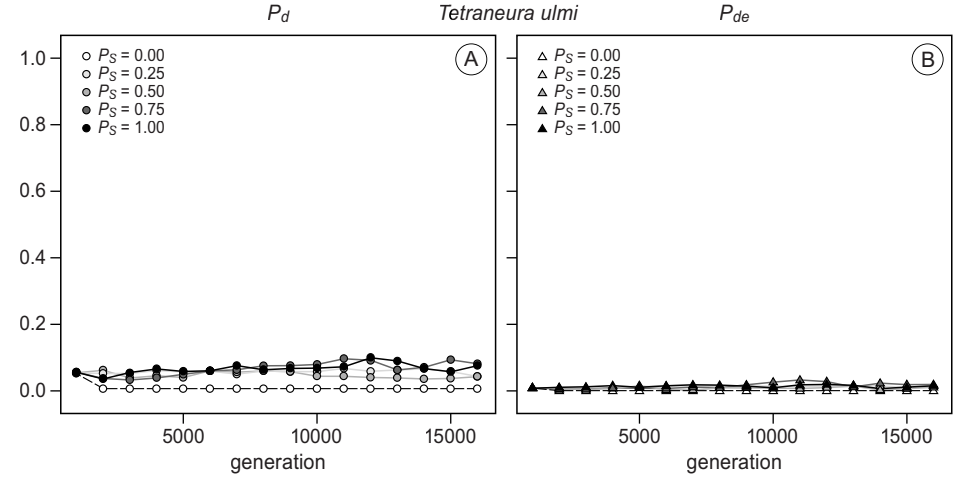


Figure 3.A1 Clonal diversity P_d (A) and the effective clonal diversity P_{de} (B) over simulation time for varying frequencies of sexual reproduction. Simulations were run for root aphid *Tetraneura ulmi*. Plots give P_d and P_{de} for 15000 generations, for five frequencies of sexual reproduction P_s (see legend). In all simulations dispersal probability P_{disp} was 0.01 and maximal dispersal distance D_{dist} was 1.

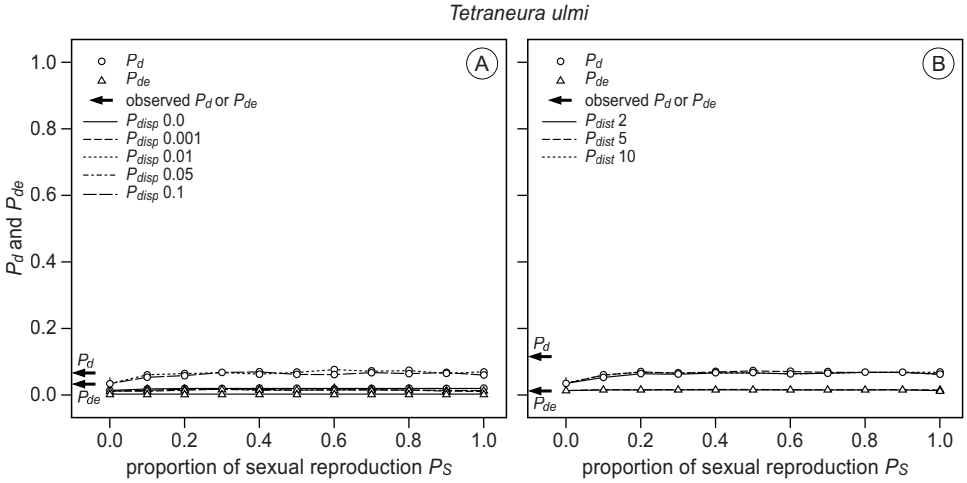


Figure 3.A2 Clonal diversity P_d and effective clonal diversity P_{de} under increasing rate of sexual reproduction P_s for various dispersal scenarios. Simulations were run for root aphid *Tetraneura ulmi*. Simulated P_d values for five dispersal probabilities and fixed dispersal distance (1) are given, and simulated P_{de} values for three dispersal distances and fixed dispersal probability (0.01). Error bars represent 95%-confidence intervals over five replicate simulations. Arrows indicate the observed estimates for P_d and P_{de} in the sampled population of *T. ulmi*.

CHAPTER 4

Ants farm subterranean aphids mostly in single clone groups – an example of prudent husbandry for carbohydrates and proteins?

Aniek B.F. Ivens
Daniel J.C. Kronauer
Franz J. Weissing
Jacobus J. Boomsma

Published in *BMC Evolutionary Biology*: 2012, **12**: 106

ABSTRACT

Mutualistic interactions are wide-spread but the mechanisms underlying their evolutionary stability and ecological dynamics remain poorly understood. Cultivation mutualisms in which hosts consume symbionts occur in phylogenetically diverse groups, but often have symbiont monocultures for each host. This is consistent with the prediction that symbionts should avoid coexistence with other strains so that host services continue to benefit relatives, but it is less clear whether hosts should always favor monocultures and what mechanisms they might have to manipulate symbiont diversity. Few mutualisms have been studied in sufficient genetic detail to address these issues, so we decided to characterize symbiont diversity in the complex mutualism between multiple root aphid species and *Lasius flavus* ants. After showing elsewhere that three of these aphid species have low dispersal and mostly if not exclusively asexual reproduction, we here investigate aphid diversity within and between ant nest mounds.

The three focal species (*Geoica utricularia*, *Forda marginata* and *Tetraneura ulmi*) had considerable clonal diversity at the population level. Yet more than half of the ant mounds contained just a single aphid species, a significantly higher percentage than expected from a random distribution. Over 60% of these single-species mounds had a single aphid clone, and clones tended to persist across subsequent years. Whenever multiple species/clones co-occurred in the same mound, they were spatially separated with more than 95% of the aphid chambers containing individuals of a single clone.

L. flavus 'husbandry' is characterized by low aphid 'livestock' diversity per colony, especially at the nest-chamber level, but it lacks the exclusive monocultures known from other cultivation mutualisms. The ants appear to eat most of the early instar aphids, so that adult aphids are unlikely to face limited phloem resources and scramble competition with other aphids. We suggest that such culling of carbohydrate-providing symbionts for protein ingestion may maintain maximal host yield per aphid while also benefitting the domesticated aphids as long as their clone-mates reproduce successfully. The cost-benefit logic of this type of polyculture husbandry has striking analogies with human farming practices based on slaughtering young animals for meat to maximize milk-production by a carefully regulated adult livestock population.

BACKGROUND

Mutualistic symbioses are widespread and of crucial importance in many ecosystems (Stachowicz 2001). Although evolutionary theory to explain the stability of mutualistic interactions has progressed considerably (see Leigh 2010 for a review), consensus on the general underlying mechanisms that keep these interactions stable and cooperative has not been achieved (Herre *et al.* 1999; Edwards *et al.* 2006; Weyl *et al.* 2010; Kiers *et al.* 2011; 2011). While further theoretical work might alleviate this problem, these difficulties also illustrate that mutualistic interactions are highly variable in their ecological contexts (Bshary & Grutter 2006; Kiers & Denison 2008; Palmer *et al.* 2010) and degrees of commitment (Poulsen & Boomsma 2005; Thompson & Fernandez 2006; Moran *et al.* 2008), and that very few of them have been studied in considerable depth (reviewed in Leigh 2010). Two aspects are thought to have important implications for the interaction stability of host-symbiont mutualisms: 1. The level of sexual reproduction and the degree of independent dispersal of the symbionts, and 2. Genetic diversity among symbionts of a single host (Herre *et al.* 1999). In a previous study we investigated the first aspect in the hitherto poorly studied mutualism of *Lasius flavus* ants farming root-aphids (Ivens *et al.* 2012b; Chapter 2). The present study focuses on the second aspect.

In cultivation (farming) mutualisms, the host partner promotes the growth of a symbiont that it consumes, either individually or as somatic modules (Hata & Kato 2006). While scenarios of 'enslavement domestication' have been suggested for the early evolution of such mutualisms (West *et al.* 2007; Login *et al.* 2011), it remains difficult to understand how symbionts would be actively selected to make the transition from free-living to being domesticated. The latter state would imply becoming reproductively isolated from free-living relatives which would require consistent direct benefits to be sustainable. Domestication often also implies losing options for horizontal transmission, having many offspring consumed by the host, and potentially being mixed with other symbiont lineages, consequences that could all discourage life as a symbiont. Domestication mutualisms would thus seem most likely to evolve if symbiont services ultimately benefit the reproduction of close symbiont relatives and if the productivity of domesticated reproduction consistently exceeds the fitness that can be obtained from a free-living life-style. When symbionts are already clonal before domestication, one would therefore expect symbioses to elaborate this form of propagation when making symbionts commit irreversibly to a dependent life-style, which requires new host-serving adaptations that impede survival and reproduction without the host. The 'trophobiotic organs' evolved in the aphids of our present study (Heie 1980; Hölldobler & Wilson 1990) are examples of such adaptations.

While symbiont interests in being cultivated would be expected to benefit from monopolizing host attention to a group of close relatives, hosts should not necessarily favor the same tendencies towards rearing monocultures, as a more variable community of symbionts might offer a broader spectrum of services or be less vulnerable to parasites (*e.g.* van Borm *et al.* 2002). As outlined by in earlier studies (Wulff 1985; Frank 1996), hosts would be selected to enforce monocultures only if scramble competition between multiple symbiont strains would decrease the overall productivity of the symbiotic interaction, *i.e.* if different symbiont strains would compete for the same limited resource provided by the

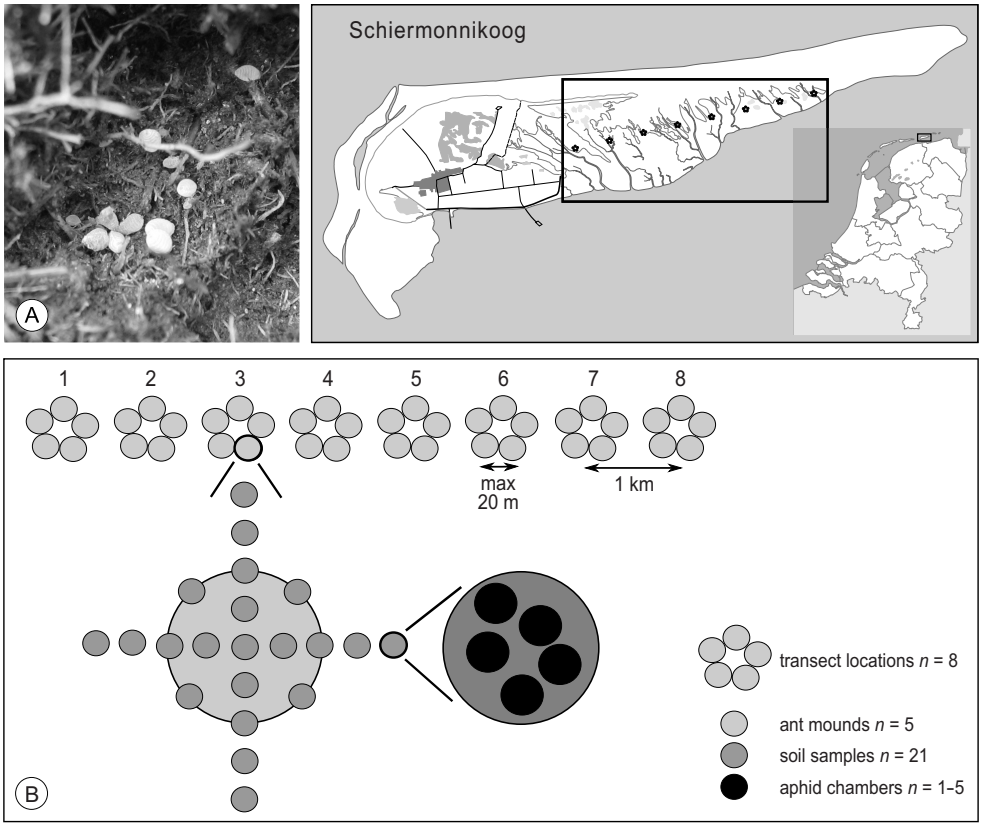


Figure 4.1 The sampling scheme for root aphids in nest mounds of the ant *Lasius flavus*.
A. A representative large aphid chamber with many, mostly adult, *Geoica utricularia*, B. Aphids were sampled from ant mounds on the island of Schiermonnikoog (The Netherlands) along a transect on the salt-marsh (framed area on map, corresponding to the area shown in Figure 4.2). Sampling was done in a nested design with four levels. At every transect location (level 1, location 1-8), we sampled 5 ant mounds (level 2), by taking 21 soil samples (level 3), located in, on the edge of, or just outside an ant mound. The collected aphids within each sample were kept separate per aphid chamber (level 4) (Photo: A.B.F. Ivens, maps courtesy of D. Visser).

host. Similar selection pressure towards monoculture farming would apply if coexistence of multiple strains within the same host would allow free-riding by underperforming strains, leading to a direct reduction in overall productivity (*e.g.* Bronstein 2001; Kiers & Denison 2008).

Incentives for competition or cheating would destabilize mutualistic interactions between symbionts and hosts, unless specific mechanisms of symbiont screening upon admission (Archetti *et al.* 2011) or symbiont rewarding/sanctioning in proportion to performance (Kiers & Denison 2008; Weyl *et al.* 2010) can evolve. The relative importance of these mechanisms is controversial, but available data suggest that monocultures are commonly found in the cultivation mutualisms that have been studied, from the gardens of

algae-growing damselfish (Hata & Kato 2002) to those of fungus-growing termites and ants (Bot *et al.* 2001; Poulsen & Boomsma 2005; Aanen *et al.* 2009; Mueller *et al.* 2010). In fungus-farming leaf-cutting ants, monocultures appear to be enforced by a combination of incompatibility between genetically different symbiont strains and active symbiont policing by the hosts (Poulsen & Boomsma 2005; Ivens *et al.* 2009; Mueller *et al.* 2010), whereas a simple mechanism of positive frequency-dependent propagation within established colonies appears sufficient to enforce life-time commitment between a termite host colony and a single symbiont clone (Aanen *et al.* 2009). However, more studies are needed to establish the generality of this principle, particularly for cultivation mutualisms where hosts are able to segregate symbionts in space or time to avoid competition (Palmer *et al.* 2010), so that the benefits of polyculture might surpass the costs.

In the present study we focus on a farming symbiosis that has been known for decades but has rarely been studied: the root aphid husbandry for sugar (honeydew, 'milk') and nitrogen ('meat') of the Yellow meadow ant *Lasius flavus*, which is likely to be essential for ant colony growth and reproduction, and involves an entire array of root aphid species (Pontin 1958; Muir 1959; Pontin 1961b; 1978; Heie 1980; Godske 1991; Seifert 2007). These root aphid species have a number of distinct traits that improve performance as ant symbionts but are never found in free-living aphids, such as the 'trophobiotic organ' to hold honeydew for the ants (Hölldobler & Wilson 1990). The most common species have further lost most if not all sexual reproduction in Northwest Europe, but have maintained low frequencies of winged morphs that may disperse between colonies (Ivens *et al.* 2012b; Chapter 2). In the present study we use a newly developed set of DNA microsatellite markers (Ivens *et al.* 2011; Chapter 5) to assess aphid species number and clonal diversity at the level of single ant nest mounds.

The objectives of our study were to use hierarchical sampling (Figure 4.1) and DNA microsatellite analysis to: 1. Estimate species- and clone diversity for three focal species of root aphids (*Geoica utricularia*, *Tetraneura ulmi*, *Forda marginata*) within *L. flavus* nests, soil samples within nests, and single aphid chambers (Figure 4.1A) within these soil samples, 2. Evaluate whether the observed distributions are consistent with the expectation that symbiont diversity within nests is low, 3. Analyze the extent to which diversity patterns change across sampling levels and years, and 4. Infer which potential mechanisms can lead to the observed diversity patterns.

RESULTS

Aphid diversity and abundance

As shown in Figure 4.2, considerable aphid diversity existed along the sampled 7 km transect, but the distribution of this diversity across ant mounds deviated significantly from random. At all sampling levels (ant mound, soil sample and chamber) monocultures containing only a single species occurred much more often than expected from a random distribution (Figure 4.2, Table 4.1), with 52% of the sampled mounds and 99% of the aphid chambers containing only a single species. Also genetic diversity within species was always non-randomly distributed over the mounds, as there were more mounds that

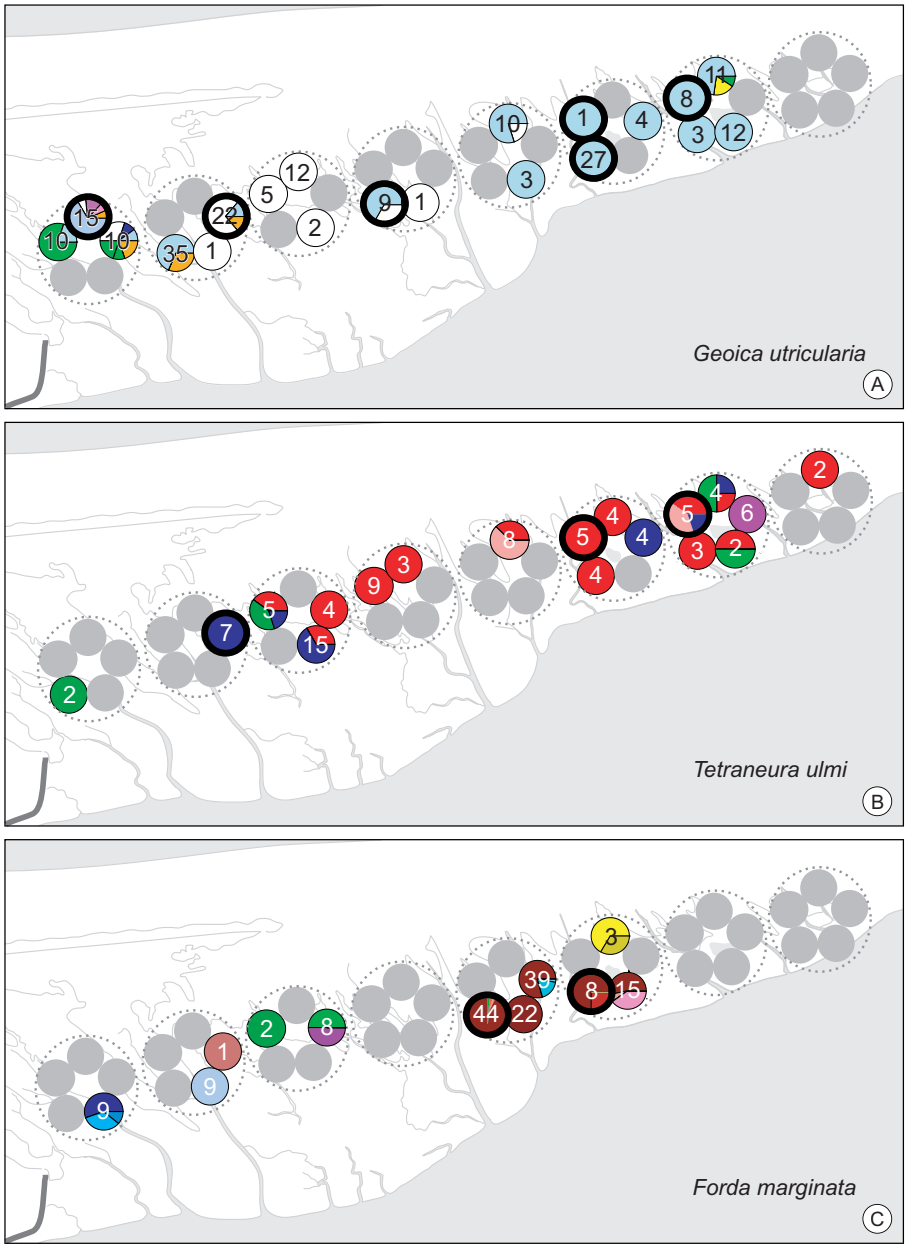


Figure 4.2 Distribution of aphid clonal lineages per ant mound. Data are shown for three root aphid species *Geoica utricularia* (A), *Tetraneura ulmi* (B) and *Forda marginata* (C) in 2008. Large dotted circles refer to sampling locations (1-8 from left to right), whereas small filled circles refer to sampled ant mounds, with the number of aphids found in the mound indicated by numbers within circles. Colours indicate the proportion of aphids belonging to particular clonal multilocus genotypes (MLGs), whereas multilocus lineages (MLLs) that combine closely related MLGs are identifiable by their similar colour shades. Mounds with bold black margins were resampled in 2009 and 2010.

contained a single multilocus lineage (MLL) than expected based on the distribution of MLLs over transect locations (Figure 4.2, Table 4.1). The same was true for the distribution of multilocus genotypes (MLGs) over mounds, with *G. utricularia* MLGs occurring significantly more often in monocultures than expected. In the other two species the frequency of MLG-monocultures across mounds was not significantly different from random expectation (Figure 4.2, Table 4.1).

At lower sampling levels within mounds (soil samples, chambers) high percentages of monocultures were also found, both between and within species (Table 4.1). However, these monoculture percentages did mostly not significantly deviate from randomness, because low aphid diversity at the species, MLL or MLG level across mounds or soil samples will automatically lead to low aphid diversity at the next level below. Figure 4.3 illustrates this for the spatial distribution of *G. utricularia* MLGs in one of the nests of Figure 4.2, showing that most MLGs occurred spatially separated already at the soil sample level, so that aphid chambers could only contain monocultures (Table 4.1, Figure 4.3). Figure 4.4 shows the distribution of aphid numbers per chamber, with most chambers containing only one aphid, but some chambers having as many as 13 aphids (means per chamber ± SE *G. utricularia* 1.61 ± 0.13, *T. ulmi* 1.84 ± 0.18, *F. marginata* 2.39 ± 0.28). Even aphid chambers with rather many aphids often contained monocultures in terms of MLLs (Figure 4.4), and chambers that did contain polycultures never had more than 2 MLLs.

Table 4.1 Results of the monoculture analyses. For each organization level (between-species and between MLLs and MLGs within-species) the probability (P) that the observed number of monocultures at a given sampling level (ant mounds, soil samples and aphid chambers) could have resulted from a random distribution of aphids was estimated using a bootstrap approach with 1000 iterations. P-values below 0.05 (bold figures) indicate deviations from a random distribution.

Level	Ant mounds			Soil samples			Chambers		
	n	% mono-cultures	P	n	% mono-cultures	P	N	% mono-cultures	P
Between species	31	52	**0.001**	145	94	**0.001**	239	99	**0.001**
Within species MLL									
Geoica utricularia	20	60	**0.028**	75	88	**0.005**	125	95	0.949
Tetraneura ulmi	18	72	**0.043**	39	90	0.068	50	96	1.000
Forda marginata	11	64	**0.015**	40	88	0.094	66	95	0.663
Between species MLG									
Geoica utricularia	20	60	**0.027**	75	88	**0.002**	125	95	0.962
Tetraneura ulmi	18	67	0.082	39	87	0.056	50	94	1.000
Forda marginata	11	36	0.099	40	73	0.707	66	88	1.000

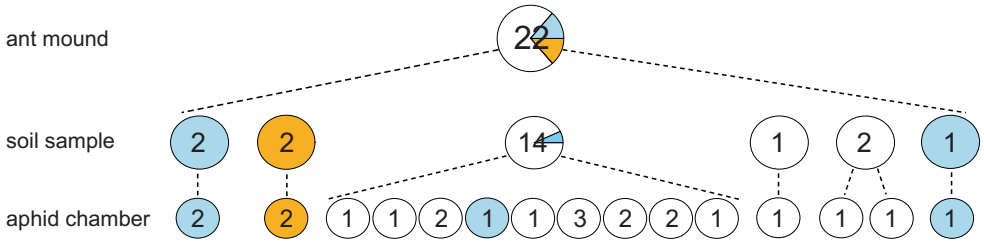


Figure 4.3 Spatial distribution of *Geoica utricularia* MLGs in a single, representative nest mound of *Lasius flavus*. The top pie chart gives the observed MLG distribution in the entire mound, the mid-level pie charts give the MLG distribution over soil samples, and the lower pie charts give the MLG distributions over nest chambers. Numbers indicate sample sizes per unit.

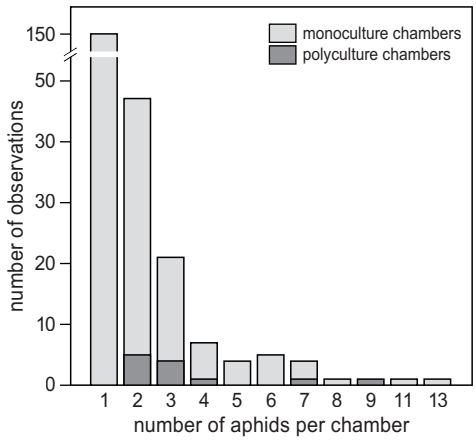


Figure 4.4 Distribution of aphids over aphid chambers in *Lasius flavus* mounds in 2008. Aphid numbers per chamber with genetic monocultures, *i.e.* aphids of the same MLL (light grey bars) and polycultures, *i.e.* chambers with aphids of multiple MLLs (dark grey bars) (n = 239). Chambers with only a single aphid are monocultures by default.

Annual turnover of aphid clonal lineages

Ten of the ant mounds sampled in 2008 were resampled in 2009 and 2010. In seven of these we found one or more of the focal species in the subsequent years (Figure 4.5). Most MLGs that we found in later years had already been found in the same mounds in 2008. There were only two exceptions to this apparent continuity over time: in the first mound resampled for *T. ulmi* (Figure 4.5B) we found an additional MLG in 2009 that had not been observed in that mound in the previous year, and in the second mound resampled for *F. marginata* (Figure 4.5C), we found a MLG that had not been identified before, but which belonged to one of the MLLs that had been observed in 2008 in other nest mounds nearby (coloured in green shade, Figure 4.2). These apparent exceptions might either reflect recent colonization events or might be due to under-sampling in 2008. For example, the

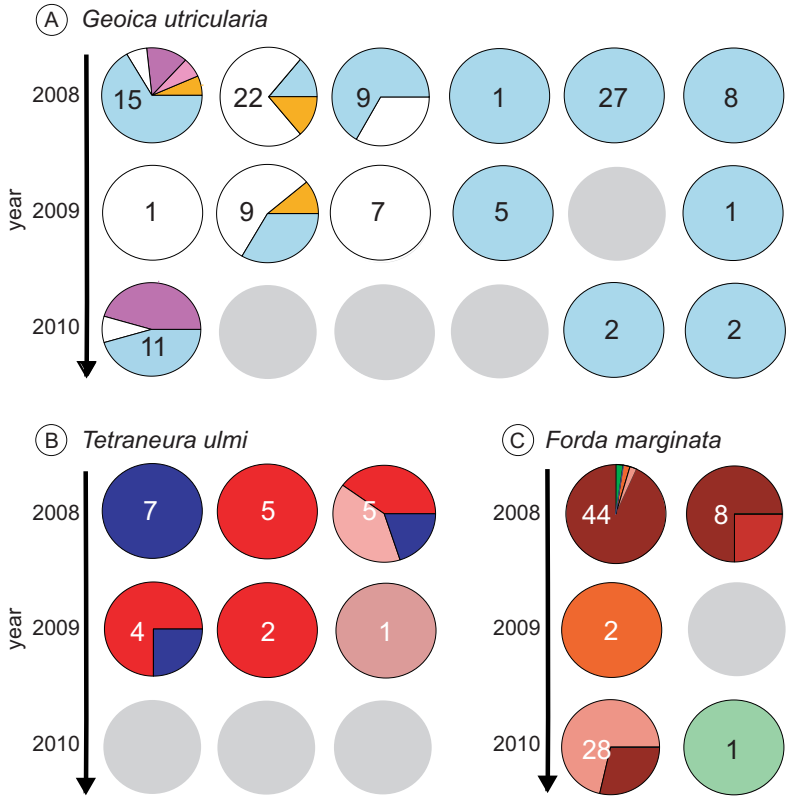


Figure 4.5 Temporal variation of aphid clones in *Lasius flavus* mounds over three years (2008, 2009, 2010). *Geoica utricularia* (A), *Tetraneura ulmi* (B), and *Forda marginata* (C). Colours indicate MLGs and correspond to colours used in Figures 4.2 and 4.3. Grey circles refer to ant mounds where a focal species was not sampled in a particular year. Data are presented for those mounds in which the same species was found in at least two of the three consecutive years.

overall composition of the nest mound in which the *F. marginata* MLG was newly observed did not change significantly between 2008 and 2009 (Fisher Exact Test, P = 0.111), likely because the newly observed MLG belonged to a MLL that had a population-wide frequency of 0.044 in 2008. With such a low frequency, it is quite likely that this MLG was missed in an earlier year. In contrast, the overall composition of aphid MLGs in the nest where we found a new MLG for *T. ulmi* did significantly change between 2008 and 2009 (Fisher Exact Test, P = 0.024). It thus appears less likely that the new MLG was due to under-sampling in 2008, since this MLG occurred at a high frequency overall (0.52). Overall, we infer that clonal lineage composition of aphid livestock in *L. flavus* ant mounds changes relatively little from year to year. We would have liked to test this with a formal heterogeneity analysis across years, but too low numbers in several cells precluded this.

DISCUSSION

Aphid distribution and abundance

In half of the ant mounds sampled in 2008 only one of the three focal root aphid species, *G. utricularia*, *T. ulmi* or *F. marginata* was found, despite the other aphid species being present within a radius of 50 m. This level of aphid specificity among ant nests matches earlier findings by Muir (1959) in a British field survey of the same ant species and its underground aphids. If there were multiple aphid species per mound, we found that they tended to be clustered in separate soil samples and hardly ever occurred in the same aphid chamber. This not only applied for the three most abundant species that we focused on, but also for other rarer species of root aphids. We are confident, therefore, that inclusion of these other aphids would not have changed our overall conclusions. Unfortunately, aphid sample sizes remained low for most mounds and for two of the three focal species, which seems unavoidable as earlier non-destructive large-scale surveys obtained similar numbers of adult root aphids for these species (Table 4.A1). Comparing frequencies and absolute densities across studies is not easy as authors have used different sampling methods in the past (Pontin 1978; Godske 1992). While these have given very different estimates of root aphid density, we show in the appendix that this is almost certainly due to these sampling differences, and that our estimates of adult aphid densities are in line with previous studies. In spite of these sample size limitations, we are confident, therefore, that our results would be repeatable with larger sample sizes at: (1) the mound level, because aphids were generally found scattered throughout the entire mound, so that systematic bias in our non-destructive sampling appears unlikely, and (2) at the chamber level, because Figure 4.4 shows that within-chamber aphid diversity does not increase with increasing numbers of aphids per chamber (ca. equivalent to chamber size). Moreover, in our statistical analysis we control for any effect of the low sample sizes, by simulating the exact same sample sizes as achieved in the field.

The considerable interaction-specificity, often between single ant colonies and single aphid lineages was also encountered at the genetic level within species. Mounds often only harbored one clonal lineage of a single aphid species and if mounds had multiple aphid clones they were almost always compartmentalized in different chambers. A similar degree of host specificity has also been shown for above-ground aphids tended by ants as opposed to non-tended aphids (Yao 2010). However, complete spatial separation of aphid clones is less frequently observed above ground (Yao 2010; Vantaux *et al.* 2011a), probably because these aphids can more easily move around. Our limited sampling across years further indicated a high degree of constancy of distributions of aphid clones over time. It would have been interesting to compare our results with similar studies on other myrmecophilous and non-myrmecophilous root aphids, but to our knowledge such studies have not yet been done.

Within- and between ant mound aphid distribution patterns

The between- and within-mound distribution patterns were very similar for the three aphid species under study (Figure 4.2), suggesting that similar dispersal and recruitment mechanisms apply. Most aphid chambers contained only a single aphid (Figure 4.4) and chamber

sizes seemed proportional to the number of aphids housed in them (appendix). It thus appears unlikely that these aphids competed for limiting phloem resources, even in the few cases where different aphid species or MLLs shared a chamber. Rather, the husbanding ants seem to optimize the feeding conditions for each aphid adult, because aphid densities (on average 1.00 per liter soil, appendix) remained well below densities that would occupy all available root phloem resources. These relatively low numbers of adult aphids can be explained by the ants eating the vast majority of aphid nymphs and only keeping a small number of adults for honeydew production as inferred previously by Pontin (1978).

The low aphid diversity per mound, the apparent invariance of clonal distributions per mound among years, and the high degree of population viscosity (Ivens *et al.* 2012b; Chapter 2) are consistent with horizontal transmission of aphids between mounds being infrequent. After successful dispersal and adoption, aphid propagation within mounds would then mostly be in the form of clonal copies of fundatrices (aphid 'foundresses') replacing their ancestors. We would thus expect that the genetic diversity of aphid livestock within a given ant mound would slowly increase over the years. The densest *L. flavus* populations in Northwest Europe are normally found in extensively grazed old pastures that have been stable for centuries and where nest-mounds are large because many generations of *L. flavus* colonies have contributed to building them. Compared to such populations, the coastal transect that we studied is more variable in age and stability, which appeared to be reflected in the younger parts of the salt-marsh harboring less aphid diversity, at least for *G. utricularia* (appendix). Patterns like this would be reminiscent of older trees having richer communities of underground mycorrhiza and leaf-endophytes (Arnold & Herre 2003; Palfner *et al.* 2005), but also of above-ground aphid colonies becoming more genetically diverse over the season due to the immigration of new aphid clones (Vantaux *et al.* 2011a).

Inferring the evolutionary logic of aphid husbandry in *Lasius flavus* colonies

Genetic diversity of symbionts has been a central issue in mutualism theory (Wulff 1985; Frank 1996; Herre *et al.* 1999) as diversity levels that simultaneously maximize the fitness of both hosts and symbionts are often expected to be low (Wulff 1985; Frank 1996). This is indeed what we found throughout our data set (*i.e.* at the species, MLL and MLG level). Compartmentalization of symbionts is known to promote mutualism stability in other systems [*e.g.* mycorrhizal mutualisms (Bever *et al.* 2009)], because benefits can be preferentially allocated toward cooperative symbionts. However, many of these conceptual arguments are based on the assumption that symbiont lineages compete and that the collateral damage of such interactions for host fitness maintains selection to suppress symbiont diversity (Frank 1996). While the high root aphid densities per *L. flavus* mound reported in the literature (appendix) that inspired this study suggested that such competition might also apply in this system, our results prompt us to reappraise this assumption, because: (1) Aphid husbandry is special, relative to other resource enhancing mutualisms, in that the ant hosts can exploit their aphid symbionts both for sugars ('milking' adults in their prime age) and for proteins (eating young instars and old adults) and (2) Our data suggest that consumption of most of the aphid offspring by the ants reduces total aphid numbers per mound (appendix) to such extent that the grass-root phloem resource constraints that might have induced aphid competition are unlikely to apply.

Many details of the interaction between *L. flavus* ants and their communities of mutualistic root aphids remain unknown and deserve further study. However, our present results indicate that the biological details and specific resource constraints of an obligate mutualism may be decisive for the selection factors that determine evolutionary stability over time. Our present data indicate that prevailing paradigms of partner choice and sanctions (Noë & Hammerstein 1994; Kiers & Denison 2008; Weyl *et al.* 2010; Archetti *et al.* 2011) may not apply in the ant-aphid mutualism that we studied, because fundamental assumptions of scramble competition between unrelated symbionts (Frank 1996) are not fulfilled (appendix).

After initial domestication, the aphid clones would have continued to benefit from the symbiosis, because the premature death of most early instar nymphs (which individually are of low value as sugar providers for the ants) reduces competition over resources and reproduction, and extensive clonality ensures that vertical transmission will maintain clonal tenure within nests. This interpretation might explain why *L. flavus* is reputedly obligately dependent on root aphids (Pontin 1978; Seifert 2007), but without having specialized on any of the large number of aphid species that are available, despite the aphids having evolved specialized traits that enhance productivity as ant symbionts but preclude independent life (see Ivens *et al.* 2012b; Chapter 2 for details). Testing the validity of our interpretation that early instar aphids are worth more as direct sources of protein than as later sources of carbohydrates will require controlled lab experiments, which might be feasible in spite of the challenges of keeping these ants and aphids in artificial nests (Smart 1991).

Analogies with human subsistence farming

The results of our study suggest that polyculture aphid husbandry in *L. flavus* follows similar efficiency principles as modern cattle husbandry practices in humans, where adult cows are kept in numbers that secure maximal milk-productivity in a competition-free environment and where surplus reproduction is slaughtered for meat-consumption soon after birth. How this analogy could come about is interesting to evaluate.

The English name for *L. flavus*, Yellow meadow ant, indicates lack of pigmentation because the ants are almost never exposed to direct sunlight. This exclusively underground life style, shared by many but far from all *Lasius* ants (Seifert 2007), must have implied that foraging territories became limited to the direct nest environment, so that access to prey was reduced but protection and monopolization of domesticated aphids became easier. Intensification of aphid husbandry thus seems a logical consequence of becoming subterranean and a prudent way of harvesting a small local resource-base that ultimately depends on primary production (grass roots) rather than secondary production (free-living prey capture). Extensive culling of immature aphids for meat not only allowed polyculture practices (by eliminating competition), but may have actively encouraged it when different aphid species would exploit somewhat different plant root niches, when their availability would be unpredictably, or when they would produce honeydew with slightly different chemical composition (Fischer & Shingleton 2001).

The analogies between aphid husbandry in *L. flavus* and human cultural practices are quite striking as farming husbandry allowed human populations to sustain themselves at

much higher densities than hunter-gatherer populations (Larsen 1995). Likewise, the density of *L. flavus* ants in mature grasslands is among the highest known for ants (Odum & Pontin 1961; Boomsma & Van loon 1982; Seifert 2007) and appears to be sustainable with only a modest ecological footprint. As in humans, the secret of success appears to be a unique combination of traits, such as the ability to actively engineer nest mound habitat [a form of niche construction (Laland & Boogert 2010)] rather than living in fixed plant structures as other obligately aphid-dependent ants do (*e.g.* van Borm *et al.* 2002), and the availability of multiple aphids that could be domesticated without the need to specialize on any one of them. This suggests that ant farming practices for meat (Offenberg 2001; Ben-Dov & Fischer 2010) deserve more explicit study, as they may provide remarkable insights into sustainable farming practice.

CONCLUSIONS

Farming mutualisms are highly diverse. Some have a long history of coadaptation, specificity and vertical symbiont transmission, whereas others have evolved interdependences based on horizontal symbiont acquisition and low specificity. Many ant species obtain facultative benefits from tending aphids. Some of these interactions have evolved to be highly specific, but the *Lasius flavus* husbandry system that we studied is unusual in that both ants and root aphids appear to be obligately interdependent and adapted to their respective life styles as farmers and livestock, but without obvious signs of species-by-species interaction specificity.

Our genetic explorations of a large island population with dense populations of *L. flavus* suggest that the combination of permanently underground nesting, aphid clonality, and very low gene flow between aphid populations of neighbouring mounds has allowed these ants to evolve an unusual form of polyculture symbiosis. Species and clonal lineages of aphids appear to be kept apart, which likely gives colonies the possibility to actively manage the diversity and abundance of their livestock. We hypothesize that this allows the ants to secure maximal yield from a subset of mature aphids that are kept for carbohydrates under optimal conditions of phloem feeding and ant care. These selected aphids may then also reproduce at the highest possible rate, so that the ants both secure maximal protein intake by eating the excess of early instar aphids, and replacement of their honeydew-producing livestock when adult aphids age and become less productive.

Many mechanistic details that govern the dynamics of this mutualism await further research. However, we feel that analogies with human husbandry practices based on similar cost-benefit considerations lend sufficient credibility to our interpretations to generate novel interest into natural selection processes that have produced ant farming practices for both meat and carbohydrates.

METHODS

Natural history of the model system

The subterranean Yellow meadow ant *Lasius flavus* constructs conspicuous nest mounds (↕ ca. 30cm, Ø ca. 80 cm) in grassland habitats to house both its own colonies and the root aphids on which it depends for honeydew as a source of carbohydrates (Pontin 1958; 1961b; 1978; Heie 1980) and which they eat for protein (Pontin 1958; 1961b; 1978). The ants actively protect the aphids (Pontin 1959; A.B.F. Ivens, personal observation) and keep them in specially constructed 'aphid-chambers', cavities around grass-roots with one or several aphids (Figure 4.1A). Thirteen species of root aphids are known to be tended by *L. flavus*, often with multiple species in the same nest mound ((Muir 1959; Pontin 1978; Heie 1980; Godske 1991; A.B.F. Ivens, personal observation). Among these, *Tetraneura ulmi*, *Geoica utricularia* and *Forda marginata* are often the most dominant species (Muir 1959; Pontin 1978; Heie 1980; Godske 1991; Ivens *et al.* 2012b; Chapter 2). This was also the case at our study site, so we focused our study on these three species. These aphids can also be found in nests of other ants, such as *Myrmica* sp. and other *Lasius* species (Heie 1980), albeit in lower numbers than in the typical *L. flavus* mounds.

Aphid reproductive cycles can be fully asexual (anholocyclic) or include a single sexual phase at the end of the season (holocyclic). In another study (Ivens *et al.* 2012b; Chapter 2) we showed that the three focal aphid species are predominantly if not completely asexual in our study population (see also below), consistent with all three species having been shown to feed year-round on roots of the grasses *Festuca rubra*, *Agrostis* spp. and *Elytrigia maritima* without requiring a host shift during winter (Muir 1959; Pontin 1978; Heie 1980; Ivens *et al.* 2012b; Chapter 2). The possible winter host shift to *Ulmus* trees that has previously been described for *Tetraneura ulmi* (Heie 1980; O.E. Heie, personal communication) thus appears to be absent in our NW European study population. However, several other mechanisms can account for more limited horizontal aphid dispersal in salt march habitats such as our study site: walking, floating on tidal floods and wind dispersal of winged individuals (alates) that are produced at very low frequencies in all three species. Considerable genetic population viscosity confirms that horizontal dispersal between mounds is generally limited (Ivens *et al.* 2012b; Chapter 2). However, this appears to be the only dispersal mode available as neither vertical nor horizontal transmission by the tending workers has ever been observed for *Lasius* ants.

Sampling methods

Ant mounds were sampled for aphids on the island of Schiermonnikoog, the Netherlands (53°28' N, 6°09' E) in July 2008, 2009 and 2010 along a 7 km transect across most of the salt-marsh on the island (Figure 4.1B). The westernmost first kilometer close to the inhabited part of the island was grazed by cattle, whereas the remaining transect crossed ungrazed salt-marsh. The transect was subdivided into eight locations (one every km). At every location we sampled five same-sized ant mounds (Ø ca. 60 cm), taking 21 cylindrical soil samples (10 cm deep and Ø 8 cm, volume 0.64 l), according to a fixed sampling scheme (Figure 4.1B). The average volume of the part of the mounds that was suitable for aphids (*i.e.* had roots of the appropriate grasses) was 66.7 l (appendix). We obtained this

estimate by adding the volume of the aboveground part of an average mound and the volume of a ring directly surrounding the mound (10 cm wide, 8 cm deep) which is known to often contain root aphids as well (Pontin 1978; Godske 1991).

Every soil sample was hand-sorted for 'aphid chambers', cavities containing one or more root aphid individuals in spatial isolation from any other aphids (Figure 4.1A). This sampling scheme resulted in a four-level nested design: transect location, ant nest mound, soil sample and aphid chamber (Figure 4.1B). In July 2009 and 2010 we resampled 10 of the 40 previously sampled nest mounds, for which we had obtained sufficiently detailed aphid distributions in 2008 to be able to detect changes in later years.

Molecular methods and data analysis

A detailed description of the molecular analysis of the aphids and properties of the genetic markers is provided by Ivens *et al.* (2011; Chapter 5). In short, all collected aphids were genotyped for an array of polymorphic microsatellite markers (*Geoica utricularia*, eight markers: Gu2, Gu3, Gu5, Gu6, Gu8, Gu9, Gu11, Gu13; *Forda marginata*, seven markers: Fm1, Fm3, Fm4, Fm6, Gu6, Gu11, Gu13; *Tetraneura ulmi*, six markers: Tu1, Tu2, Tu3, Tu4, Tu10, Tu11) after DNA extraction from entire aphids using 200 μl 20%-Chelex® 100 resin (Fluka) (Walsh *et al.* 1991). Following PCR-amplification, products were analyzed on an ABI-PRISM 3130XL (Applied Biosystems) sequencer and chromatograms were analyzed in Genemapper (Applied Biosystems).

When amplification failed, samples were re-run at least two more times. When amplification remained unsatisfactory, the specific microsatellite locus was scored as 'missing data'. When data were missing for more than half of the loci the individual was omitted from further analysis. In total, we included 239 individuals of *Geoica utricularia* (2008: 201, 2009: 23, 2010: 15, after omitting a total of 28 individuals), 191 of *Forda marginata* (2008: 158, 2009: 4, 2010: 29, 11 omitted) and 105 of *Tetraneura ulmi* (2008: 92, 2009: 7, 2010: 6, 4 omitted).

Diploid clonal multilocus genotypes (MLGs) consist of a unique combination of alleles across all genotyped loci. The genotypic data allowed us to assign every aphid to a MLG using the software MLGSIM 2.0 (http://www.rug.nl/fmns-research/theobio/downloads), an updated version of MLGSIM (Stenberg *et al.* 2003b; Box B). A multilocus lineage (MLL) is a group of closely related MLGs that differ by only one or two alleles (Ivens *et al.* 2012b; Chapter 2). All MLGs could be grouped into MLLs. The complete analysis is detailed in Ivens *et al.* (2012b; Chapter 2).

When a sample only contained aphids from a single species, MLL or MLG, we classified that sample as a 'monoculture' at the species, MLL or MLG level. Samples were taken at three 'sampling levels': ant mound, soil sample or chamber. To test whether the observed monocultures occurred more frequently than expected under a random distribution, we wrote a bootstrap routine in R 2.13.0 (R Development Core Team 2011) (routine available upon request). For a given level of sampling, the routine distributed the species, MLLs, or MLGs randomly over samples in 1000 iterations with simulation sample sizes corresponding to the observed sample sizes. The routine thus used the observed frequency distributions of species, MLLs, or MLGs at the sampling level above the focal level (Figure 4.1B) to estimate the probability (P) that the same or a higher number of monocultures than the

observed number would be obtained by chance (one-tailed test). When P was <0.05, the null hypothesis that the observed number of monocultures resulted from a random distribution of aphids over samples was rejected.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ABFI and JJB designed research with input from IP and FJW. ABFI did the fieldwork and the genetic analyses, the latter with contributions from DJCK, both for the practical work and the final analyses. ABFI conducted the statistical analyses, with contributions from IP. ABFI and JJB wrote the paper, with several rounds of contributions from DJCK, IP and FJW. All authors read and approved the final manuscript.

Authors' information

All authors have long standing interest in the evolutionary biology of cooperation and conflict within and between species. JJB, DJCK and ABFI mainly focus on different aspects of social evolution of ants and their mutualistic partners. FJW and IP are theoretical biologists, focusing on the theory of conflict and cooperation in social systems.

Acknowledgements

We thank Thijs Janzen and Sylvia Mathiasen for help in the field and in the laboratory, Søren Feodor Nielsen for statistical advice, Ruth Howison for help with the analysis of the elevation data (appendix) and David Nash and two anonymous reviewers for comments on earlier versions of the manuscript. A PhD-Grant of the Centre for Ecological and Evolutionary Studies, University of Groningen and grants by the Langerhuizen Fund and the Nicolaas Mulerius Fund, awarded to ABFI supported this study, as well as a grant by the Danish National Research Foundation to JJB. We are grateful to 'Vereniging Natuurmonumenten' for permission to do field work in the national nature reserve Schiermonnikoog.

APPENDIX

Aphid abundance estimates

The aphid numbers in our samples were low relative to the numbers that have previously been estimated for *L. flavus* territories (5500–17000 aphids per ant nest of medium size, *i.e.* 23000 ants) (Pontin 1978; Godske 1992). However, neither in previous surveys nor in the present study were mounds sampled exhaustively, in order to preserve them for later resampling. This implied that aphid density estimates could only be based on extrapolations from aphid numbers in soil core samples, which in our study covered on average 20% (range 11.4%–62.8%) of the estimated total volume of ant mounds that was suitable for aphid-culture.

We collected 14.5 ± 2.07 (mean ± s.e.) adult aphids of the focal species per nest, which produced an estimate of the cumulative total adult aphid population per mound for the three focal root aphid species of 67 individuals (range 18–134) (Table 4.A1). Most of the discrepancies with earlier estimates appear to be due to previous studies using Tullgren funnel extraction methods, so that all developmental stages were collected over a period of several days, whereas we used hand-sorting that only allowed collection of adult aphids and occasionally fourth instar nymphs (Table 4.A1). The differences in numbers obtained will likely have been further enlarged by the fact that aphids will continue to give birth during the 4-5 day long Tullgren extraction, with many of them ending up in the collection vials instead of being eaten by the ants (Pontin 1978; Smart 1991). Moreover, earlier authors included a larger part of the potential ant territory and sampled ten root aphid species more than we were able to analyse genetically. Approximate corrections for these possible sampling biases produced density figures of adult aphids per litre of mound-soil that were much closer to our present findings (Table 4.A1).

On average 88% of the root aphids that previous authors collected by Tullgren funnel extraction belonged to the nymphal stages that our hand sampling method missed. It therefore seems reasonable to assume that almost all of these never become established in chambers as carbohydrate providers to the ant society, but were eaten by the ants before they become adult (Pontin 1978). This would imply that population numbers of adult root aphids remain well below the density levels that would exploit all available phloem resources that could possibly be accessed via grass roots. Whether *L. flavus* indeed balances its preying behaviour based on carbohydrate intake would need further testing in controlled laboratory experiments (Pontin 1978). A result consistent with this hypothesis would seem likely, because a shift from milking to preying behaviour has been documented for *L. niger* after workers were offered a carbohydrate food supplement (Offenberg 2001). *L. niger* belongs to the same genus as *L. flavus* and often lives in the same grasslands habitats where it avoids competition with *L. flavus* by foraging above ground (Pontin 1961a; 1963; Boomsma & Van loon 1982).

Further arguments for the likely absence of scramble competition between root aphids

Of the total of 239 opened aphid chambers that were inhabited by the three focal species in 2008, only 92 (38.5%) contained more than one aphid (range 2–13). Within this subsample only a single chamber contained aphids of two species and only 11 chambers

Table 4.A1 Observed and estimated number of aphids per litre of soil in sampled mounds of *L. flavus* in different studies.
Observed numbers of aphids per liter of soil inferred from the available literature are given only for July and August, as that period corresponds to the sampling scheme applied in the present study. We estimated percentages of observed adults of *G. utricularia*, *T. ulmi* and *F. marginata* after correcting original total numbers (Pontin 1978; Godske 1991; 1992) by the cumulative percentage of these three species relative to all root aphids (older studies found 4-5 more species, which we ignored) and by adjusting for the percentage of adults (close to 100% in our study and much less in the other studies). This produced the final estimates of the number of adult aphids of these three species per liter of soil and per mound, showing that numbers are roughly comparable.

Study	Observed number of aphids/liter soil (summer)	% focal species	% adults	Corrected number of aphids/liter soil		Estimated mound volume (l)		Original estimated total number of aphids/mound	Corrected estimated total number of aphids/mound	
				Mean	Range	Mean	Range		Mean	Range
Pontin 1978	32.9[1]	53.3	8.4	1.48	-	85.00	-	12700	125.80	-
Godske 1992	48.3	28.7	15.0	2.08	-	56.00	-	5506	116.48	-
Present study	1.73[2]	63.9[2]	92.1[2]	1.00	0.86 - 1.14	66.76	21 - 117	-	66.99	18.06 - 134.12

[1]Observed numbers are given per soil sample in Pontin (1978). We estimated the volume of these soil samples to be 1.13 l and corrected observed numbers accordingly
[2]Based on adults and fourth instar nymphs only.

(4.6%) contained 2 MLLs (Figure 4.4). Aphid chambers are small cavities that are excavated by the ants alongside roots of grasses like *Festuca rubra* and *Elytrigia maritima*. Although chamber volumes were not measured, they often seemed approximately proportional to the number of aphids housed in them, suggesting that the ants expand chambers when they need to contain more adult aphids and more roots for these aphids to extract phloem sap from. Combined with the abundant availability of grass roots in *L. flavus* mounds and our average yield of ca. 1 adult aphid per litre soil (Table 4.A1), this minimal coexistence with non-clone mates and the absence of chamber space constraints would make it very unlikely that individual ant-tended aphids would not have access to ad libitum phloem resources.

External factors that may affect aphid diversity at a larger scale

Overall, we would expect that the genetic diversity of aphid livestock would tend to slowly increase when *L. flavus* mounds become larger over the years of their existence, but we did not have a range of mound-size data to test this and neither are we aware of directly relevant data on this by others. However, the transect locations that are known to be the oldest from historical records about salt marsh development on the island of Schiermonnikoog (locations 1,2 and possibly 7) (Olff *et al.* 1997), harbored mounds that yielded a higher aphid diversity, at least for *G. utricularia* for which we had most data. Similarly, mounds occurring at lower elevations will be more frequently subjected to flooding, a disturbance that might cause mounds to be growing slower and have longer periods without abundant ant habitation (Boomsma & Isaaks 1982). Also this seems at least partially consistent with our data, as aphid clonal diversity in mounds on transect locations with lower elevation levels [*e.g.* location 5 and 6 (Ivens *et al.*, unpublished data), tended to have lower clone diversity with the exception of *F. marginata*]. Aphid numbers of each of the three species varied considerably across the transect, possibly reflecting subtle differences in local ecological conditions related flooding frequency and salinity owing to slight elevation differences (Ivens *et al.*, unpublished data). None of these differences appear to have affected the overall results and conclusions of our study, but they may be of interest for future ecological studies of *L. flavus* populations in coastal areas.

BOX A

Partner choice in a farming mutualism: do ants display preference for certain homopteran symbionts?

Aniek B.F. Ivens
Ido Pen
Franz J. Weissing
Jacobus J. Boomsma

ABSTRACT

Mutualism, cooperation between members of different species, is assumed to be prone to conflicts between partners about investments and returns. One of the mechanisms that can aid alignment of these interests is partner choice: the selective directing of resources towards preferred partners or the neglect of unwelcome partners. Partner choice requires the ability to discriminate among potential symbionts. Here, we studied the ability of ant workers of *Lasius flavus* to preferentially select among its obligate homopteran symbionts relative to control homopterans that are not tended. In this pilot experimental set-up we observed sub-colonies of ants that were offered mealybugs and aphids that originated from their own colony or from neighbouring ant colonies as well as symbionts belonging to different species, all in different states (dead/alive), in different developmental stages (nymph/adult) or belonging to different clonal genotypes. We show that the acceptance rate of symbionts did not differ among any of the categories evaluated. Total interaction time of the ants with the symbionts was, however, significantly shorter for nymphs and one of the genotypes, and also for the mealybugs. In fact, the mealybugs offered were largely ignored by the ant workers, consistent with them never being actively tended by *Lasius flavus* in the field. We use these preliminary results to evaluate whether the low number and clone diversity of aphid species in ant mounds may be caused by passive aphid dispersal constraints, rather than by active aphid selection 'at the gate'.

INTRODUCTION

Mutualisms are ubiquitous in nature (Leigh 2010), but their evolutionary stability is still not completely understood. How can mutualisms remain stable interactions over evolutionary time, when cooperating partner species are likely selected to reap maximal benefits from the interaction for the least possible investments (Herre *et al.* 1999)? Several mechanisms to prevent or resolve such conflicts and thus enhance mutualism stability have been put forward in the literature. One of these, partner choice (Bull & Rice 1991) allows (one of) the species to choose with whom to cooperate by preferentially directing benefits. If cooperative individuals tend to choose cooperative partners, the potential for conflict over cooperative investment will ultimately be reduced. This mechanism has been studied both theoretically (*e.g.* Noë & Hammerstein 1995; Archetti *et al.* 2011) and empirically (*e.g.* Bshary *et al.* 2002; Kiers & Denison 2008; McFall-Ngai 2008). Partner choice requires discriminatory power in the choosing species, which prompted us to study the discriminatory power of *Lasius flavus* ants when confronted with different underground homoptereans.

Work on partner choice in homopteran-tending ants has so far been restricted to a couple of studies on facultative interactions between ants and above ground aphids. These studies showed that aphid species honeydew composition induces a preference hierarchy for the aphids species tended, with species producing melezitose-rich honeydew being preferred (Völkl *et al.* 1999; Woodring *et al.* 2004). However, Vantaux *et al.* (2012) showed that *Lasius niger* ants do not appear to discriminate in tending or predation of high and low quality clones of aphids.

Here we present pilot study results on partner choice in the obligate interaction between the underground ant *L. flavus* and the homopterans it tends in its nests. *L. flavus* is well-known for rearing several aphid species (Paul 1977; Pontin 1978; Godske 1991). Also mealybugs are frequently found in the nests of these ants, but their relationship to the ants has remained unclear, although mealybugs are often described as being also mutualistic partners of ants for honeydew (Hölldobler & Wilson 1990). In an experimental subcolony set-up we investigated ant preferences for both underground aphids and mealybugs belonging to different species, and for aphids from the ants' own colony or a different colony, for different developmental stages, different clonal lineages, and different states (dead or alive) and measured possible preferences in terms of symbiont acceptance rate and total interaction time.

METHODS

Study system

The subterranean Yellow meadow ant *Lasius flavus* houses root aphids and mealybugs in its conspicuous nest mounds(↕ ca. 30 cm, ø ca. 80 cm) (Paul 1977; Pontin 1978; Godske 1991). The ants depend on these symbionts for honeydew as a source of carbohydrates and they are also known to eat the root aphids for protein (Pontin 1958; Pontin 1961b; Pontin 1978; Smart 1991). Aphids are kept in specially constructed 'aphid-chambers', cavities around grass-roots with one or several aphids and are actively protected from preda-

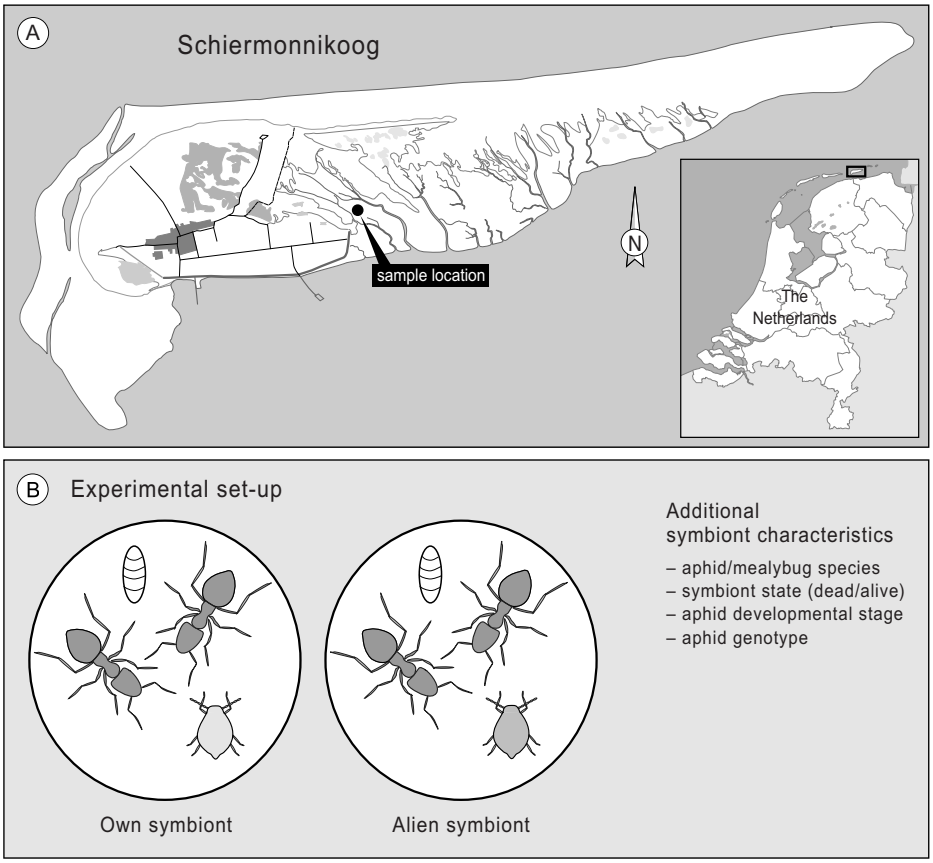


Figure A.1 Sampling location and experimental set-up. (A) Ant colonies used in this study were located at the ungrazed salt marsh of the island of Schiermonnikoog in The Netherlands. (B) experimental set-up of behavioral experiments. Each sub-colony consisted of two *Lasius flavus* worker ants and one ant pupa. These sub-colonies were offered either one of their own symbionts (aphid or mealybug) or an alien symbiont, collected from another ant mound (Maps courtesy of D. Visser).

tors by the ants. Thirteen species of root aphids are known to be commonly tended by *L. flavus*, often with multiple species in the same nest mound (Paul 1977; Pontin 1978; Godske 1991; Chapter 8), but rarely in the same chamber (Ivens *et al.* 2012a; Chapter 4). Among these, *Tetraneura ulmi*, *Geoica utricularia* and *Forda marginata* are often the most dominant species. Besides aphids belonging to these four species, also *Forda formicaria* aphids were used as well as aphids belonging to the genera *Anoecia* and *Trama*. Also several species of mealybugs have been found in *L. flavus* nests (Chapter 8). Not all mealybugs used in this study could be identified; those who were identified belonged to the species *Rhizoecus albidus* Goux or *Euripersia tomlini* (Newstead). *E. tomlini* (Newstead) has earlier been described by Kosztarab & Kozar (1988) to be associated with *Lasius flavus*. However, the exact relationship between these mealybugs and the ants remains unknown.

Experimental set-up

Experiments were performed directly after collection of ants and aphids on the island of Schiermonnikoog, the Netherlands (Figure A.1A) in July 2010 and 2011. For each sampled ant colony (n = 14), we made a series of experimental sub-colonies following Bot *et al.* (2001) and Ivens *et al.* (2009). Sub-colonies were housed in a small plastic pot (↕ 3.5 cm, Ø 2 cm) with a lid with 15 pinholes to provide air. Each sub-colony consisted of two ant-workers, soil from the original ant nest and a pupa to secure natural nursing behavior by the workers (Figure A.1B). After set-up, the sub-colonies were allowed to acclimatize during 2 hours in complete darkness.

Sub-colonies were then offered a symbiont collected from their own colony or from a neighboring colony (maximum distance between colonies 10 m). For each symbiont a set of characteristics was recorded for later reference: (1) Symbiont origin (own colony/ alien colony), (2) symbiont 'species' (when possible to determine: normally aphid species, otherwise aphid genus; mealybugs where merely classified as 'mealybug'), (3) symbiont state (dead/alive) and symbiont developmental stage (adult/nymph). A total of 134 symbionts were tested. After the experiments *Geoica utricularia* and *Forda marginata* aphids were genotyped using microsatellite markers (*Geoica utricularia*, eight markers: Gu2, Gu3, Gu5, Gu6, Gu8, Gu9, Gu11, Gu13; *Forda marginata*, seven markers: Fm1, Fm3, Fm4, Fm6, Gu6, Gu11, Gu13). All details of the protocol for this genotyping analysis can be found in Ivens *et al.* (2011; 2012a; 2012b; Chapters 2, 4 and 5).

Behavioral observations

After introduction of the symbiont individual, each sub-colony was observed for 5 minutes. All observations were video-recorded for later reference and seven behaviors were recorded and timed: A: antennation of the symbiont by the ant worker, *LA*: licking of the abdomen of the symbiont by ant worker, *LB*: licking of the back of the symbiont by ant worker, *M*: 'milking', the consumption of a droplet of honeydew from the symbiont by the ants, *P*: picking up of the symbiont (mostly to be carried to the brood item, presumably a safe location), *D*: dropping of the symbiont, and I: symbiont ignored by the ants throughout the experiment. A and LA are standard behaviours in both the milking and the recognition processes in ants (Hölldobler & Wilson 1990).

Data analysis

We analyzed two major response variables: (a) symbiont acceptance by the ants and (b) total interaction time between ants and the symbionts. A symbiont was considered 'accepted' when behaviors *M*, *P* or *L*, and *A*, occurred, the latter two only when in combination with behavior *M*. Because consistent exact timing of all behaviors was not feasible (mostly problematic when aphids were carried out of view), total interaction time was estimated from all behaviors observed: *A*: 5 seconds, *A* in combination with *M*: 10 seconds, *BL*: 10 seconds, *I*: 0 seconds, *LA*: 10 seconds, *LA* in combination with *M*: 10 seconds and *M*: 10 seconds. These estimates of time spent were all based on time observations during the experiments.

Our primary interest was to investigate how symbiont acceptance and symbiont interaction time varied with different aphid characteristics. Acceptance rate was analyzed using

a generalized linear model (GLM) with acceptance rate as response variable and symbiont origin, 'species', state and developmental stage as explanatory factors. Significance of factors was tested separately and statistical interactions were not considered. As small datasets with many more zeroes than ones do not allow for reliable testing using standard GLM models, we followed Firth's modified score procedure in logistic regression analysis using R-package Logistf (Firth 1993). These model tests yield estimates for the penalized maximum likelihood (or 'Firth-Logistic' (FL)) after which factor effects are evaluated for statistical significance using a χ^2-test. Because symbiont genotype data were only available for the aphid species *G. utricularia* and *F. marginata*, the effect of genotype was tested in a separate GLM for each species, following the same procedure as for the full model.

Total interaction time plus one second was log-transformed to improve normality and analyzed using analysis of variance with total interaction time as response variable and symbiont origin, 'species', state and developmental stage and their interactions as explanatory factors. Post-hoc testing (for 'species') was performed using the R-package multcomp (Hothorn *et al.* 2008). The effect of genotype was tested separately for *G. utricularia* and *F. marginata* with total interaction time as response variable and genotype as explanatory factor. All analyses were performed using R 2.13.0 (R Development Core Team 2011).

RESULTS

Symbiont acceptance rates

Overall acceptance rates turned out to be very low as 83% of the offered symbionts remained unaccepted (Figure A.2). Analysis of the full model comprising all symbiont characteristics available (symbiont origin, symbiont 'species', symbiont state and symbiont developmental stage) showed that none of these characteristics significantly affected symbiont acceptance rate (Table A.1; Figure A.2). Apparently, ants do not discriminate among symbionts from their own nest and symbionts from other nests, among different symbiont 'species', among dead or living symbionts, and among different developmental stages when accepting aphids into their nest. Mealybugs were, however, never accepted.

Table A.1 Analysis of variance for acceptance rate and total interaction time. Shown are the final model (bold) after model simplification and the full model without interactions (plain text). Df denotes degrees of freedom of predictor variables, Δ FL the change in penalized maximum likelihood (for acceptance rates) and *F* (*F* test, for total interaction time) and *P*, the statistical significance upon removal of the predictor. Significant P-values ($P < 0.05$) are given in bold.

		Acceptance rate		Total interaction time	
Source	**Df**	**Δ FL**	***P***	***F***	***P***
Symbiont species	6	7.023	0.32	7.20	**< 0.001**
Symbiont developmental stage	1	0.003	0.96	7.17	**0.008**
Symbiont state	1	0.840	0.36	2.54	0.11
Symbiont origin	1	1.507	0.22	0.56	0.34

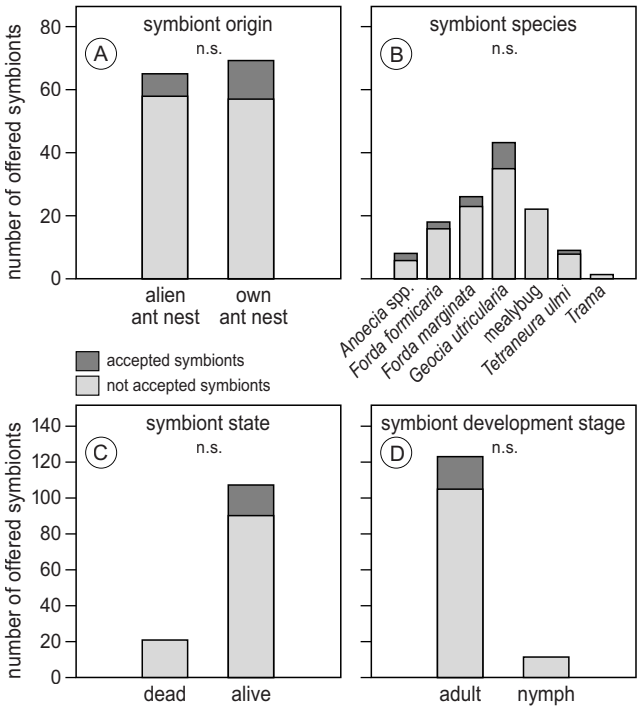


Figure A.2 Acceptance rate of offered symbionts. Number of accepted symbionts (dark grey) and not accepted symbionts (light grey) plotted for different symbiont characteristics: Origin of the symbiont (own ant nest/alien ant nest) (A), symbiont 'species' or group (B), symbiont state (dead/alive) (C) and symbiont developmental stage (adult/nymph) (D). None of the factors had a significant effect on acceptance. In total 134 symbionts were offered. n.s.: $P > 0.05$.

Total interaction time between ants and symbionts

Total interaction time was also not significantly affected by symbiont origin and state (Table A.1), but symbiont 'species' and developmental stage did have a significant effect, with nymphs having a shorter interaction time than adults and mealybugs having a shorter interaction time than aphid symbionts (Table A.1; Figure A.3). There were no significant differences among aphid species in interaction time, but mealybugs were interacted with shorter (and rejected more quickly) than all aphid species/genera tested.

Effect of genotype

We did not observe differences in acceptance rate among *G. utricularia* and *F. marginata* genotypes (GLM, *G. utricularia*: df = 2, ΔFL = 1.31, $P = 0.27$, *F. marginata*: df = 2, ΔFL = 1.02 and $P = 0.36$; Figure A.4). However, we did find a difference in total interaction time among *G. utricularia* genotypes as G2 aphids were handled significantly longer than G3 aphids (effect of 'genotype' in *G. utricularia*: $F(2,29) = 4.36$ $P = 0.02$; Figure A.4C). In *F. marginata*, we did not observe differences in total interaction time among the 3 genotypes ($F(2,18) = 1.71$ $P = 0.21$; Figure A.4D).

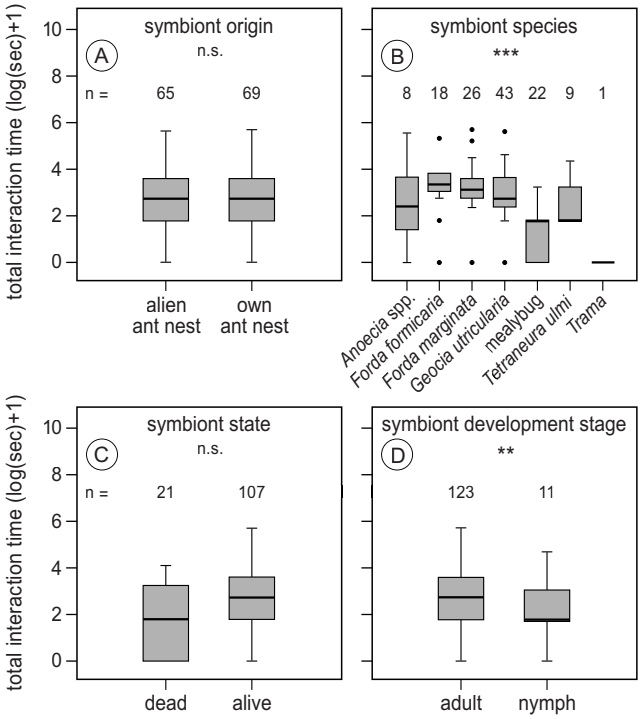


Figure A.3 Box-whisker plots of total interaction time between symbionts and ants. Logarithm of total number of seconds (+1) ant workers spent interacting with the offered symbionts, plotted for different symbiont characteristics: Origin of the symbiont (own ant nest/alien ant nest) (A), symbiont species or group (B), symbiont state (dead/alive) (C) and symbiont developmental stage (adult/nymph) (D). Solid lines show medians, boxes show their 25-75 percentiles and whiskers the interquartile range. Not all outliers are shown to retain clarity. Only symbiont 'species' and developmental stage had a significant effect on total interaction time, with mealybugs and nymphs being interacted with significantly shorter than other symbionts. n.s.: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

L. flavus ants prefer aphids over mealybugs

Ants spent significantly more time interacting with aphids than with mealybugs. Total interaction time included carrying aphids around, antennating them and trying to "milk" them, behaviours that were practically never expressed towards the mealybugs. In addition, not a single mealybug was accepted into the sub-colony and carried next to the brood item, as often happened to accepted aphids. These mealybugs are frequently found in *L. flavus* nest mounds and are presumed to be honeydew providing symbionts for *L. flavus* ants, although very little is known about this interaction (Kosztarab & Kozár 1988). However, our results seem to indicate that *L. flavus* does not actively tend these mealybugs, nor prey on them. Instead, the mealybugs may be commensalistic inhabitants of ant nests, which provide a safe environment with relatively constant temperature and humidity.

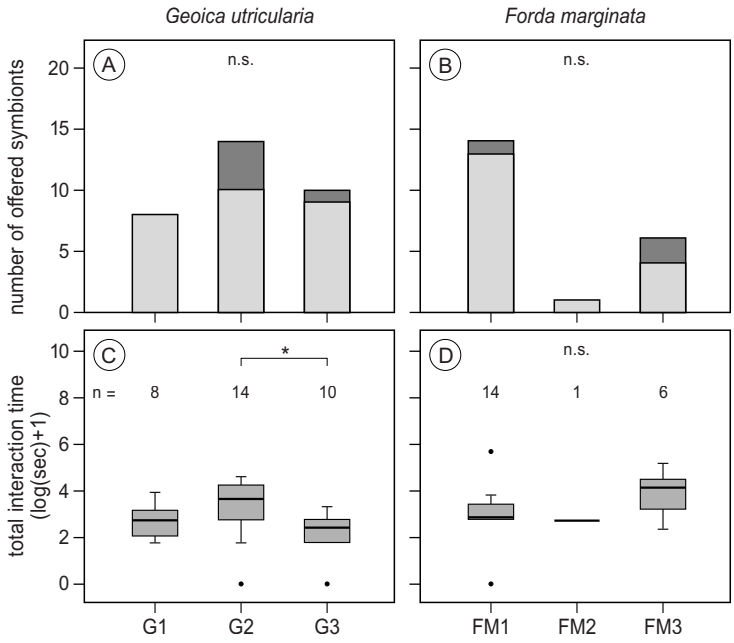


Figure A.4 Acceptance rate of and total interaction time with three clonal lineages of aphids *Geoica utricularia* and *Forda marginata*. *G. utricularia* (A, C) and *F. marginata* (B, D) acceptance rate (dark grey: accepted, light grey: not accepted) and total interaction time are given for three clonal lineages of each species (*G. utricularia*: G1, G2, G3, *F. marginata*: FM1, FM2, FM3), determined by microsatellite analysis. Within-species differences in acceptance rate of clones were tested to be non-significant. Within-species differences in total interaction time with ants between clones were non-significant in *Forda marginata*, but in *G. utricularia* G2 was interacted with significantly longer than G3. n.s.: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Ant discrimination among aphid species and genotypes

Our results show that ants do not differ in acceptance rate of or total interaction time with different aphid species/genera. Aphid species are known to differ in honeydew quality and quantity and previously above ground ants have been shown to differentiate among the aphid species that they facultatively tend (Völkl *et al.* 1999; Woodring *et al.* 2004). In our set-up we observed only a couple of times that milking occurred, so experimental ants may not have obtained sufficient information on honeydew quality or quantity to actively prefer certain aphids during the experimental time given them. Also the honeydew produced by different clonal genotypes belonging to a single aphid species is known to differ in composition (Vantaux *et al.* 2011b). However, our results did not show differences in acceptance rate among aphids clonal lineages, except that one *G. utricularia* clone was interacted with longer than another clone, indicating that some discriminatory power may exist in the ants after all. Vantaux *et al.* (2012), however, showed that *Lasius niger* ants did not distinguish

between aphid clones of the same facultative aphid species, even though the honeydew produced differed in quality. To be able to assess whether similar dynamics apply to an obligate, underground ant-aphid interaction, future experiments on *L. flavus* preference of aphid species and clones should therefore include longer assessment times for the ants, recently fed experimental aphids, and the performance of chemical analysis of the honeydew produced by the different aphids.

Symbiont state and developmental stage

L. flavus ants rely on aphids for both carbohydrate and nitrogen intake, as aphid honeydew provides sugar and aphid predation yields proteins (Pontin 1958; Pontin 1961b; Pontin 1978; Smart 1991; Ivens *et al.* 2012a; Chapter 4). Aphids of different developmental stages presumably have a different nutritional destination for the ants: the majority of the nymphs has been inferred to be eaten by the ants, whereas adult aphids are preserved for honeydew production (Pontin 1978; Ivens *et al.* 2012a; Chapter 4). Likewise, one might expect that dead aphids can still serve as food. To test whether ants distinguish between aphids that can serve as different food sources, we offered both nymphs and adults as well as dead and live aphids. The results show that ants do not prefer living over dead aphids in terms of acceptance rate or interaction time. However, ants do spend significantly more time interacting with adult aphids than nymphs (including milking and depositing aphids next to the brood), suggesting that the ants may have at least some discriminatory power.

No effect of symbiont origin

Symbiont recognition by ant hosts has previously been shown in fungus growing ants which distinguish their own resident symbiont fungus from alien fungus and even police against the latter, helped by the resident fungus (Bot *et al.* 2001; Poulsen & Boomsma 2005; Ivens *et al.* 2009), a form of discrimination that could be a mechanism to ensure long-term host-symbiont association. The tested *L. flavus* ants, however, did not differ in acceptance rate or interaction time with symbionts originating from their own nest or from neighbouring nests. This suggests that the ants do not distinguish between these two groups of symbionts and may thus not be able to recognize their own “dairy cattle”. This is surprising, because the ants may transfer colony-specific cuticular hydrocarbons (CHC) when antennating their symbionts, which one would expect could label them as part of the colony (Hölldobler & Wilson 1990). This would be consistent with the observation that ants display very similar behavior towards their aphids as towards their brood (Way 1963; personal observation). *L. flavus* ants display low aggression levels (A.B.F. Ivens, personal observation), which could possibly be correlated with less pronounced CHC-profiles and thus lower discriminatory power among the different profiles. Future CHC-analyses of ant workers and their associated aphids could further elucidate this.

Implications of results and future perspectives

The experiments shown here served as preliminary studies of partner choice in the *L. flavus* – root aphid farming mutualism. The pilot character of the experiments caused the sample sizes to be rather unbalanced and some factors causing variation might not have been controlled for (*e.g.* temperature, weather conditions, distance between sampled colonies). Nevertheless, these experiments showed that partner choice experiments in this system are

feasible and they yielded a couple of interesting preliminary results. Overall, the ants show very little discriminatory power between aphids. This suggests that the low aphid species and genetic diversity observed within single ant mounds may not be caused by active ant screening upon entry into a colony, but rather be due to aphid dispersal constraints which implies that local clones are seldomly being "diluted" by immigrants (Ivens *et al.* 2012a; Chapter 4).

Acknowledgements

The authors would like to thank Maurice Jansen for mealybug species identification, Marten van de Sanden for help with the experimental equipment, and Saleta Perez Vila and Sebastian Baldauf for statistical advice. This study was supported by a PhD-Grant of the Centre for Ecological and Evolutionary Studies, University of Groningen and grants by the Langerhuizen Fund and Nicolaas Mulerius Fund, awarded to A.B.F.I as well as a grant by the Danish National Research Foundation to J.J.B.

CHAPTER 5

Characterisation and cross-amplification of polymorphic microsatellite loci in ant-associated root-aphids

Aniek B.F. Ivens
Daniel J.C. Kronauer
Jacobus J. Boomsma

Published in *Conservation Genetics Resources*: 2011, 3: 73-77

ABSTRACT

Twenty-six polymorphic microsatellite loci were developed for four species of ant-associated root-aphids: *Geoica utricularia*, *Forda marginata*, *Tetraneura ulmi* and *Anoecia corni*. We found up to 9 alleles per locus, with an average of 4.8. We also report polymorphic cross-amplification of eleven of these markers between different pairs of study species. Furthermore, we tested previously published aphid microsatellites and found one locus developed for *Pemphigus bursarius* to be polymorphic in *G. utricularia*. These microsatellite markers will be useful to study the population structure of aphids associated with the ant *Lasius flavus* and possibly other ants. Such studies are relevant because: 1. *L. flavus* mounds and their associated flora and fauna are often key components in protected temperate grasslands, and 2. *L. flavus* and its diverse community of root-aphids provide an interesting model system for studying the long-term stability of mutualistic interactions.

Mutualistic interactions between species are widespread and play key roles in ecosystem stability and diversity (Stachowicz 2001; Bastolla *et al.* 2009). In Northwest Europe, the Yellow meadow ant *Lasius flavus* keeps up to fourteen species of mutualistic root-aphids in its nests (Pontin 1978; Heie 1980; Godske 1991). The ants actively tend the aphids, which provide them with honeydew (Pontin 1978). The nest mounds are markers of high grassland biodiversity and long-term habitat stability (Dean *et al.* 1997; Blomqvist *et al.* 2000; Lenoir 2009). However, despite the decline of European temperate grasslands in recent decades and the associated losses in plant and invertebrate biodiversity (WallisDeVries *et al.* 2002), neither the sociobiology of the ants (but see Boomsma *et al.* 1993), nor the biology of the root-aphids (Pontin 1978; Godske 1991; 1992) have been extensively studied. To facilitate molecular ecological approaches in the study of this mutualism, we developed DNA microsatellite markers for the four commonest species: *Forda marginata*, *Tetraneura ulmi*, *Geoica utricularia* and *Anoecia corni*.

Samples for genomic library construction for *Forda marginata*, *Tetraneura ulmi*, and *Anoecia corni* were collected in 2007 from an ant-nest on the Dutch island of Schiermonnikoog (53º29'03.5"N; 6º13'46.1"E) whereas *Geoica utricularia* was collected near Dejret, Denmark (56º12'54.2"N; 10º24'48.2"E). All samples for molecular analysis were preserved in 96% ethanol.

Genomic DNA was extracted using the QIAGEN DNeasy Blood & Tissue kit and enriched for poly-CA and poly-CT microsatellite containing fragments using the protocol by Rütten *et al.* (2001). We designed PCR primers for the flanking regions of repetitive motifs using the web-based software Primer 3 (Rozen *et al.* 2000).

Primers were tested on Schiermonnikoog samples collected in 2007, 2008 and 2009 and on samples collected near Dejret in 2007 (*Anoecia* spp.). DNA for microsatellite screening was extracted using 200 μl 20%-Chelex® 100 resin (Fluka) (Walsh *et al.* 1991). PCR-cocktails had a total volume of 10 μl, consisting of 0.8 mM dNTPs, 2 mM $MgCl_2$, 1 × PCR buffer, 0.25 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 1 μl of DNA template and a varying concentration of primers (Table 5.1). Several primer pairs were multiplexed in PCR (Table 5.1). The amplification conditions were 95ºC for 5 minutes, x number of cycles of 95ºC for 30 sec., T_a for 30 seconds and 72ºC for 30 seconds (1 minute for Gu3, Gu8, Gu9, Gu10 and Gu13) and a final extension of 15 minutes at 72ºC. The respective x and T_a for each primer are listed in Tables 5.1 and 5.2.

Amplified fluorescent labeled PCR-products were run on an ABI-PRISM 3130XL (Applied Biosystems) sequencer and chromatograms were analyzed in Genemapper (Applied Biosystems). Expected and observed heterozygosities and deviations from Hardy-Weinberg Equilibrium (HWE) were determined using GENALEX 6.2 (Peakall & Smouse 2006). Occurrence of Linkage Disequilibrium (LD) was assessed using Genepop 4.0 (Rousset 2008).

The fourteen markers developed for *Geoica utricularia* were tested on 5-227 aphids. All markers were polymorphic, with 5.3 alleles per locus on average (Table 5.1). The four polymorphic markers for *Forda marginata* were tested together with three cross-amplifying markers (Gu6, Gu11, Gu13) on 125-162 aphids yielding 6.0 alleles on average (Tables 5.1 and 5.2). The six microsatellite markers for *Tetraneura ulmi* had 3.7 alleles on average in 60-94 tested aphids (Table 5.1). Observed and expected heterozygosities are given in

Table 5.1 Characteristics of 26 polymorphic microsatellite loci in different species of ant-associated root-aphids. *N* number of tested samples, N_a number of alleles, H_E expected heterozygosity, H_O observed heterozygosity, T_a annealing temperature.

Locus	Species	Primer sequence (5'-3') (F: forward. R: reverse)	Repeat Motif	Size range (bp)	*N*	N_a	H_E	H_O	T_a (°C)	Nr. of cycles *x*	Primer conc. (μM)	Multiplex mix	Genbank accession number
Gu1	*Geoica utricularia*	F: ATCAAACGAACGAACCGAAT R: GCGAAAGTTATGGCGTTTGT	$(GT)_8$	113-118	5	4	0.740	1.000	50	40	0.35	Gu-3	HM582813
Gu2	*Geoica utricularia*	F: CGCGATTAGATCTCGGAATG R: AAATCGTATAAAAGTAAAGGCGTTAT	$(GT)_{11}$	158-177	227	5	0.613	0.361	50	40	0.15	Gu-2	HM582814
Gu3	*Geoica utricularia*	F: TATCGTGCGGACACAGACAT R: CGGGCTATACCGCATACACT	$(TA)_9$	192-208	169	7	0.665	1.000	50	40	0.15	Gu-1	HM582815
Gu4	*Geoica utricularia*	F: CTGCTGCTCGTCCGACTTA R: GCAGATAAAAACTGTTAGCCTTGA	$(TG)_6$ C $(AT)_{12}$	206-222	8	4	0.602	0.125	50	35	0.35	Gu-3	HM582816
Gu5	*Geoica utricularia*	F: CACAGGACGCGTAACTTAATATAG R: ACACTTTTCGGCAATTTCGT	$(GT)_{15}$	164-214	214	6	0.569	0.145	50	40	0.15	Gu-2	HM582817
Gu6	*Geoica utricularia*	F: ATCAAACGGTCTGGCATGTA R: CAATATCTCATCTGCCAGCAA	$(TG)_3$ CG $(GT)_8$	151-200	199	7	0.539	0.337	50	40	0.15	Gu-2	HM582818
Gu7	*Geoica utricularia*	F: GTTAAAGGAACTCTTACGCTCTACG R: CATATAAATAAAAACGTCCTGTAGGC	$(CA)_3$ TA $(CA)_5$	87-103	13	4	0.698	0.000	50	40	0.35	Gu-3	HM582819
Gu8	*Geoica utricularia*	F: TATACACGTCCGCGCAGATA R: GTTCGTTGCTCGTCGACTTT	$(AC)_{10}$	233-237	199	3	0.479	0.060	50	40	0.15	Gu-1	HM582820
Gu9	*Geoica utricularia*	F: CGCGCGTTATGAAAAATGTA R: CTCGCTGTGTGTGACACCTT	$(CA)_{13}$	223-250	184	8	0.800	0.799	50	40	0.15	Gu-1	HM582821
Gu10	*Geoica utricularia*	F: CGCCGCTAAAGAAGTTTTCA R: TTACGTTAAACA(AC)ACGAGGATTTAT	$(GT)_{19}$	228-261	14	8	0.763	0.786	50	40	0.35		HM582822
Gu11	*Geoica utricularia*	F: CGGTTACCCGTAAAAGGCTTA R: AAATCGCAATGACAGTCACG	$(CA)_{11}$	145-153	223	6	0.729	0.677	50	40	0.15	Gu-2	HM582823
Gu12	*Geoica utricularia*	F: GAGCCAACTGCCCGTTATAG R: CGGTTTTATTTAAGGTCTCGAA	$(GT)_{12}$ GC $(GT)_{25}$ A $(GT)_4$	106-138	10	3	0.460	0.000	60	45	0.15		HM582824
Gu13	*Geoica utricularia*	F: TCGCCGTCGACTATTTTACA R: AGTTACGTCCGGGGAGAAAT	$(CAG)_7$ $(N)_{21}$ $(TC)_{10}$	202-218	188	7	0.754	1.000	50	40	0.15	Gu-1	HM582825
Gu15	*Geoica utricularia*	F: TTTTTACGGGCTAAACCCTATTT R: CCAATACGGATCCCAACTTTT	$(GA)_{15}$ $(A)_4$ $(GA)_3$ $(A)_9$	165-167	10	2	0.180	0.200	50	40	0.25		HM582826

Table 5.1 Continued.

Locus	Species	Primer sequence (5'-3') (F: forward. R: reverse)	Repeat Motif	Size range (bp)	*N*	N_a	H_E	H_O	T_a (°C)	Nr. of cycles *x*	Primer conc. (µM)	Multiplex mix	Genbank accession number
Fm1	*Forda marginata*	F: CCTCCAATTACCGTTCAACC R: GAAGAACGTGACACGCGATA	$(TG)_{22}$ CG $(TG)_5$	182-259	154	9	0.458	0.253	53	37	0.15		HM582827
Fm3	*Forda marginata*	F: TCTGATTTTTCGTTCGTCCA R: CGCGGCTCGTTACCTATTTA	$(AT)_{10}$	225-349	138	6	0.494	0.246	50	40	0.15		HM582828
Fm4	*Forda marginata*	F: CATTACGTGTGAGTGTAATATAGTTTT R: TGGTTTAAACGACGGATTTTC	$(AC)_{14}$	178-200	162	7	0.465	0.167	50	35	0.15		HM582829
Fm6	*Forda marginata*	F: TCACTCGCCTAGCGTTATTC R: GTGGCCGTAGCATGTCACTA	$(T)_{11}$ ATGA $(T)_{23}$	250-280	125	4	0.709	0.920	50	45	0.15		HM582830
Tu1	*Tetraneura ulmi*	F: CGGGTGCGTGGGTACATTAT R: ATACGTTGAGCCAACTACCG	$(GT)_4$ GAT$(AG)_5$ T - $(GA)_{10}$ $(A)_6$ $(N)_6$ $(T)_{17}$	218-241	89	2	0.164	0.000	50	35	0.25		HM582831
Tu2	*Tetraneura ulmi*	F: TCCGACATACGTTTAACCAAAA R: ATGACACCCCTGCCACTATC	$(TA)_7$ $(TG)_8$	157-159	60	2	0.180	0.000	50	40	0.25	Tu-1	HM582832
Tu3	*Tetraneura ulmi*	F: CGCCGTAAATAATAATAACAACAA R: CACGAGACCAAGAGATAAGGAAA	$(A)_{11}$ $(AT)_6$ $(TA)_2$ $(C)_3$ $(GT)_9$	234-264	89	5	0.702	0.921	50	35	0.25		HM582833
Tu4	*Tetraneura ulmi*	F: TTATTCGCAACCACACCTTG R: ACGCGACGGATAGAAATACG	$(GT)_{26}$ G $(GT)_3$	182-203	94	6	0.636	0.904	50	40	0.25	Tu-1	HM582834
Tu10	*Tetraneura ulmi*	F: AGTATACGCGTCTCGCCAAC R: GGAGCAAGTCCGATCGTTAT	$(TAA)_3$ TGA $(TAA)_7$	233-248	87	3	0.226	0.253	50	40	0.25		HM582835
Tu11	*Tetraneura ulmi*	F: CGGAGAACGCGTATTGATTT R: CGTGCGCGTGTCAAAGTAT	$(GT)_9$ $(TA)_5$	194-200	89	4	0.396	0.393	50	35	0.25		HM582836
Ac6	*Anoecia corni*	F: CGAGGCATATTCAAAATGTAAGA R: CAGCATTAAACACGAATGCAA	$(AT)_3$ G $(TA)_9$ C $(AT)_2$	148-164	6	2	-	-	45	45	0.25		HM582837
Ac8	*Anoecia corni*	F: AATAATAATTCGTGGCGTTGC R: CGCCGTAGAAGCAAATAATATC	$(ATT)_{10}$	160	4	1	-	-	45	45	0.25		HM582838

Tables 5.1 and 5.2. Since all species reproduce asexually, deviations from HWE and presence of LD are expected (Ivens *et al.* 2012b; Chapter 2; 3). All loci indeed showed significant deviation from HWE, except for Gu15 in *Geoica utricularia*, Fm4 and Gu11 in *Forda formicaria*, and Tu10 in *Tetraneura ulmi*. In *G. utricularia* the majority of the loci pairs (65%) had significant LD, with most pairs not in LD involving Gu1 and Gu15. All pairs of *T. ulmi* were in LD, except for Tu10-Tu2, Tu10-Tu1, Tu2-Tu11 and Tu1-Tu11. In *F. marginata*, all loci pairs were in LD.

The two primer pairs developed for the genus *Anoecia* amplified across *Anoecia* species but were not extensively tested. We merely report these loci here for future reference. Cross-amplification was tested for all markers except Gu12 and Fm5 (Table 5.2), yielding eleven markers that amplified in one or more additional species. Moreover, most markers used (species specific and cross-amplified) for *Forda marginata* were also suitable for the sibling species *Forda formicaria*. The loci Fm3, Fm4, Fm6 and Gu13 proved to be diagnostic for distinguishing between *F. marginata* and *F. formicaria* (Table 5.2). Three markers from *Pemphigus bursarius* (Pb02, Miller *et al.* 2000) and *P. spyrothecae* (97PS12 and 98PS8, Johnson *et al.* 2000) were tested for cross-amplification in our focal species, but only Pb02 reliably cross-amplified in *Geoica utricularia* (Table 5.2).

Table 5.2 Cross-amplifications of microsatellite markers in different species of ant-associated root-aphids. *N* number of tested samples, N_a number of alleles, H_E expected heterozygosity, H_O observed heterozygosity, T_a annealing temperature.

Locus	Cross-amplified species	Size range (bp)	*N*	N_a	H_E	H_O	T_a (°C)	Nr. of cycles *x*	Primer conc. (µM)	Genbank accession number
Gu6	*Forda marginata*	151-176	159	5	0.681	0.672	49	40	0.15	HM582818
Gu11	*Forda marginata*	135-147	162	6	0.489	0.234	49	40	0.15	HM582823
Gu13	*Forda marginata*	143-178	159	5	0.430	0.000	45	45	0.15	HM582825
Tu11	*Forda marginata*	-	2	-	-	-	49	40	0.15	HM582836
Fm3	*Forda formicaria*	121	18	1	0.000	0.000	50	40	0.15	HM582828
Fm4	*Forda formicaria*	174-178	18	3	0.495	0.777	50	35	0.15	HM582829
Fm6	*Forda formicaria*	206-291	18	2	0.500	1.000	50	45	0.15	HM582830
Gu6	*Forda formicaria*	151-152	17	2	0.110	0.000	49	40	0.15	HM582818
Gu11	*Forda formicaria*	142-146	18	3	0.439	0.277	49	40	0.15	HM582823
Gu13	*Forda formicaria*	156	19	1	0.000	0.000	45	45	0.15	HM582825
Fm1	*Anoecia corni*, *A. zirnitsi*	110-134	7	3	-	-	45	45	0.25	HM582827
Tu2	*Anoecia corni*, *A. zirnitsi*	137-148	3	2	-	-	45	45	0.25	HM582832
Tu11	*Anoecia corni*, *A. zirnitsi*	69-126	7	5	-	-	45	45	0.25	HM582836
Ac 8	*Anoecia zirnitsi*, *A. major*	130-146	2	2	-	-	45	45	0.25	HM582838
Pb02[a]	*Geoica utricularia*	118-124	8	2	-	-	50	40	0.20	AF267192

[a]developed by Miller *et al.* 2000 for the lettuce root-aphid *Pemphigus bursarius*

Although we enriched specifically for $(CA)_n$ and $(CT)_n$ repeats, the aphid DNA appeared to be especially AT-rich, including repeats that were suitable for microsatellite design. This observation is in accordance with earlier findings (Weng *et al.* 2007).

In conclusion, the 26 newly developed microsatellite markers presented here cover a large proportion of the known root-aphid fauna associated with *L. flavus* and other ant species (Heie 1980), and will be useful for detailed studies of the ecology and evolution of this mutualistic association.

Acknowledgements

This study was supported by a PhD-Grant from the Centre for Ecological and Evolutionary Studies, University of Groningen and grants from the Pieter Langerhuizen Fund and the Nicolaas Mulerius Foundation, awarded to A.B.F.I., as well as a grant from the Danish National Research Foundation to J.J.B. that funded D.J.C.K.'s postdoctoral work in Copenhagen. The authors would like to thank Sylvia Mathiasen, Thijs Janzen and Elzemiek Geuverink for help in the laboratory and in the field, Ole E. Heie and Maurice Jansen for help with aphid taxonomy and Franz J. Weissing and Ido Pen for discussion.

BOX B

MLGsim 2.0: updated software for detecting clones from microsatellite data using a simulation approach

Aniek B.F. Ivens
Marten van de Sanden
Joke Bakker

INTRODUCTION

The ability to reproduce both sexually and asexually can be found in many organisms (Halkett *et al.* 2005). Such organisms with mixed reproductive modes include economically important species such as pest species (*e.g.* aphids) and agricultural crops (*e.g.* potato). Despite recent theoretical and empirical advances (Balloux *et al.* 2003; De Meeus & Balloux 2004; Halkett *et al.* 2005; D'Souza & Michiels 2010; Villate *et al.* 2010; Chapter 3) it remains difficult to accurately estimate rates of asexual reproduction in predominantly sexual populations from microsatellite data alone. Moreover, sexually and asexually produced individuals can be hard to distinguish, especially in plants. However, the mode of reproduction is known to have important implications for the evolutionary ecology and population genetics of a species (Balloux *et al.* 2003; Halkett *et al.* 2005; Arnaud-Haond *et al.* 2007). Detecting clonal individuals in sexual populations thus greatly contributes to a more complete insight into the evolution and ecology of a number of focal study species (Stenberg *et al.* 2003a; 2003b; Villate *et al.* 2010).

The most frequently used statistical method for assessing the likelihood that a given individual is of clonal origin from microsatellite data, is the estimation of the P_{sex} value of the multilocus genotype (MLG) to which the individual belongs. P_{sex} gives the probability to observe at least as many identical MLGs in the sampled population, as expected in a random-mating population in Hardy-Weinberg equilibrium (Tibayrenc *et al.* 1990; Parks & Werth 1993; Young *et al.* 2002):

$$P_{sex} = \sum_{i=n}^{N} \frac{N!}{i!(N-i)!} (P_{gen})^i \, (1-P_{gen})^{N-i} \tag{B.1}$$

with N = sample size and n = number of individuals with the same MLG. P_{gen} is the probability of that MLG to occur in a diploid population assuming Hardy-Weinberg equilibrium:

$$P_{gen} = \left(\prod_{j=1}^{L} (a_j b_j) \right) 2^h \tag{B.2}$$

with L = number of loci, a_j and b_j = frequencies of alleles a and b at locus j, respectively, and h = number of heterozygous loci in the sample (Parks & Werth 1993). To take into account departures from Hardy-Weinberg equilibrium using fixation index F_{IS}, a measure for heterozygote excess or deficit in the population (Weir & Cockerham 1984), a more conservative probability $P_{gen,F_{IS}}$ can be estimated (following Arnaud-Haond *et al.* 2007):

$$P_{gen,F_{IS}} = \left(\prod_{j=1}^{L} a_j b_j + c_j F_{IS,j} \right) 2^h \tag{B.3}$$

with L, a_j, b_j and h the same as above, $F_{IS,j}$ the estimated F_{IS} for locus j, and $c_j = a_j(1-a_j)$ if locus j is homozygous (*i.e.*, $a_j = b_j$) and $c_j = -a_j b_j$ if j is heterozygous (Hartl & Clark 1997).

P_{sex} values can vary between 0 and 1, with a very low value for an MLG indicating that the multiple observations of that particular MLG have a very low probability of being derived from separate events of sexual reproduction. In other words, the repeated occurrence of the MLG can be best explained by these individuals belonging to the same clonal

lineage. However, P_{sex} cannot be treated as an accurate probability that is consistent and comparable between populations, because the distribution of P_{sex} values of MLGs in a population will also depend on the allelic variation present in the population (equation (B.1)). Here, we present simulation software that allows the estimation of the critical P_{sex} value for statistical significance in a given population and thus allows for the statistical significance of observed P_{sex} values to be evaluated.

Overview of MLGsim 2.0

The software MLGsim 2.0 is an updated and extended version of MLGsim by Stenberg *et al.* (2003b). MLGsim 2.0 reads microsatellite data of diploid organisms and assigns all individuals to MLGs for which P_{sex} values are then estimated based on their counts and allele frequencies determined from the observed dataset. Next, the program simulates the sampled population a (user-specified) number of times taking the estimated allele frequencies and realized sample sizes into account. These simulations then provide a distribution of simulated P_{sex} values from which a critical P_{sex} threshold value can be determined against which the observed P_{sex} values can be statistically tested.

Since its release, the original software MLGsim has been widely used in molecular ecological and evolutionary studies on a wide variety of organisms including weevils (Stenberg *et al.* 2003a), aphids (Vorburger 2006), willows (Stamati *et al.* 2007), corals (Severance & Karl 2006) and fungi (Xhaard *et al.* 2011). However, recent developments in genomic techniques have allowed reaching much larger sample sizes than previous studies, which implied that the original MLGsim software can no longer be used as it can maximally handle 200 sampled individuals and needs manually encoded data input.

The updated version MLGsim 2.0 includes, beside bug-fixes, the following new features:

(1) no restriction to dataset sample size
(2) microsatellite data will be read automatically from a standard data file
(4) the software calculates allele frequencies directly from the data file
(5) individuals are sorted and assigned to MLG and MLG-counts are now automatised
(6) the program allows for estimation of both P_{gen} (equation (B.2)) and $P_{gen,F_{IS}}$ (equation (B.3))
(7) the software now calculates the observed and expected heterozygosities H_O and H_E and the fixation index F_{IS}.
(8) the user can specify whether allele frequencies and population genetic estimates should be calculated based on *ramet* data (all samples included) or *genet* data (each MLG included only once) (*sensu* Harper 1977)
(9) several commonly used estimates for clonal diversity are calculated for the dataset:
    - Clonal diversity and its modified counterpart $R = (G - 1)/(N - 1)$, with $G =$ number of MLGs and $N =$ number of individuals (Ellstrand & Roose 1987; Dorken & Eckert 2001; Arnaud-Haond *et al.* 2007).
    - Effective clonal diversity $P_{de} = \frac{G_e}{N}$ with effective number of genotypes G_e given by $G_e = \frac{1}{\sum g_i^2}$ in which g_i is the frequency of the i^{th} genotype (MLG)

    (Balloux *et al.* 2003; Chapter 3)

Application of MLGsim2.0

The limitation of the original software MLGsim to datasets comprising at most 200 individuals has previously been listed as a shortcoming in several studies and a review (Halkett *et al.* 2005; Vorburger 2006; Stamati *et al.* 2007). MLGsim 2.0 can in principal handle unlimited sample sizes. Since its release online, MLGsim 2.0 has already been successfully applied in several studies with large sample sizes or large numbers of loci, including studies on corals (Gorospe & Karl in review; 2352 samples, 6 loci), ants (Kronauer *et al.* in review; 83 samples, 30 loci) and aphids (Ivens *et al.* 2012b; 18 – 201 samples, 6-8 loci).

In 2006, the predecessor of MLGsim 2.0 was used to analyse population genetic data of aphids (Vorburger 2006), which necessitated using only a subsample of 200 individuals of the total sample of 365. We re-analyzed the complete dataset using MLGsim 2.0 and three of its new features [custom number of iterations, the 'FIS' option (P_{sex} estimation based on $P_{gen,F_{IS}}$ rather than P_{gen}) and the 'MLG' option (allele frequencies based on *genet data*)]. These new analyses do show a couple of quantitative and qualitative differences between previous and present results (Table B.1). The first analysis (2nd row, Table B.1), is identical to the analysis by Vorburger (2006), but now includes the complete dataset of 365 individuals rather than a subset of 200 individuals. By including all sampled individuals, the range of P_{sex} values increased from 10^{-11} – 10^{-15} to 10^{-8} – 10^{-15}, possibly making it easier to detect critical P_{sex} values for statistical significance. An increased number of iterations yielded the same results (3rd row, Table B.1). Using the newly implemented FIS option (4rd row, Table B.1) also resulted in higher significance levels for a clonal origin of MLGs. Using the newly implemented *genet data* option, in which allele frequencies are calculated based on a subset of the entire dataset comprising of only a single copy of each MLG, fewer significant P_{sex} values were observed. By using the *genet data* (5th and 6th row, Table B.1) for this dataset, fewer MLGs thus are estimated to be of clonal origin than previously reported (Vorburger 2006).

Table B.1 Re-analysis of aphid data from Vorburger (2006). The published study used a subsample of 200 individuals instead of the complete dataset to analyse the data with MLGsim using 1000 iterations. These original results are shown in the first row. We re-analysed the complete dataset with 1000 iterations using the new features of MLGsim 2.0 FIS (using $P_{gen,F_{IS}}$) and MLG (using a *genet* subsample of the data to estimate allele frequencies).

	P_{sex} range	Significance level
Vorburger 2006 (subset used)	10^{-11} – 10^{-15}	0.05
MLGsim 2.0 (1000 iterations)	10^{-8} – 10^{-15}	0.05 – 0.01
MLGsim 2.0 (10000 iterations)	10^{-8} – 10^{-15}	0.05 – 0.01
MLGsim 2.0 (option FIS)	10^{-8} – 10^{-15}	0.01
MLGsim 2.0 (option MLG)	10^{-6} – 10^{-15}	ns – 0.01
MLGsim 2.0 (options MLG, FIS)	10^{-6} – 10^{-15}	ns – 0.01

In sum, MLGsim2.0 allows for more efficient clone detection in a population genetic data set of diploid organisms based on co-dominant microsatellite markers. This updated version of MLGsim is now suited for datasets of unrestricted size, it uses automated data input, sorts individuals into MLGs and calculates their statistical significance, and provides an array of population genetic and clonal diversity estimates.

CHAPTER 6

Conditional dispersal can promote and hamper cooperation in unexpected ways

Aniek B.F. Ivens
Ido Pen
Jacobus J. Boomsma
Franz J. Weissing

ABSTRACT

Although cooperation provides benefits to all parties involved, it does not evolve easily, because cooperators can be exploited by cheaters. This problem may be overcome if cooperative individuals predominantly interact with other cooperators. Two mechanisms leading to such positive assortment are partner choice (seeking cooperative and rejecting non-cooperative neighbourhoods) and partner fidelity (staying in cooperative and leaving non-cooperative neighbourhoods). We used individual-based simulations to investigate whether partner choice and partner fidelity indeed favour the evolutionary emergence and subsequent stability of cooperation in a patch-structured population. We organized our simulations as a public goods game, where investment in cooperativeness could jointly evolve with the tendency to leave a patch to join another patch. Both dispersal and settlement decisions were allowed to be conditional on average cooperativeness in source and target patches. Conditional settlement had a negligible effect on the evolutionary outcome, but conditional dispersal had a strong effect. If the cost-benefit ratio of public good investment was very low (making any investment profitable for the investors themselves), conditional dispersal destabilized cooperation that readily evolved when dispersal was kept constant. If the cost-benefit ratio of investing in public goods was high (making the public goods game a social dilemma where investors lose while their group gains) conditional dispersal strongly promoted the evolution of cooperation, but only in simulations where individuals evolved the tendency to leave cooperative patches. In simulations where individuals tended to stay in cooperative patches (partner fidelity), cooperation quickly went extinct. This unexpected finding is explained by the fact that a high level of cooperation is associated with a high intensity of local competition in our model, making it profitable to leave cooperative environments and cash the benefits of cooperation elsewhere. Our results imply that conditional dispersal can both promote and destabilize cooperation, and that the evolutionary consequences of conditional strategies may thus be difficult to predict.

INTRODUCTION

Cooperation is found throughout nature in a wide variety of organisms ranging from microorganisms to primates (Sachs *et al.* 2004; West *et al.* 2007). Ecologically dominant organisms are often characterized by a high degree of cooperation, as exemplified by biofilm-producing microorganisms, social insects, or our own species. Yet the origin and maintenance of cooperation is not fully understood, especially in social dilemma situations where from an individual perspective cheating is more profitable than cooperation (among others Janssen & Goldstone 2006; Killingback *et al.* 2006; Lehmann & Keller 2006; Nowak 2006; West *et al.* 2007; Fletcher & Doebeli 2009; Bijma & Aanen 2010).

It is generally believed that a tendency for positive assortment between cooperative individuals is an important prerequisite for the evolution and stability of cooperation (Lehmann & Keller 2006). Already Hamilton (1964a) proposed that limited dispersal could promote the evolution of cooperation, because under limited dispersal interacting individuals will often be related and, thus, have similar tendencies to cooperate. However, limited dispersal favours the evolution of cooperation only under very specific conditions (Queller 1992; Taylor 1992; Queller 1994; West *et al.* 2002; Kümmerli *et al.* 2009). This is because limited dispersal will not only increase the scope for cooperation with kin, but also for competition with relatives (Taylor 1992; Kümmerli *et al.* 2009).

Still, dispersal could have an important effect on the evolution of cooperation if it is not random but conditional on the cooperativeness in the local environment. Partner choice and partner fidelity are important aspects of such conditional dispersal. As argued by Bull and Rice (1991), the evolution of cooperation should be strongly promoted if individuals preferentially settle in cooperative neighbourhoods (partner choice) and if they only stay in those neighbourhoods where the level of cooperation is sufficiently high (partner fidelity). Several modelling studies have confirmed these predictions. In an iterated prisoner's dilemma game, Sherratt and Roberts (1998) showed that including the option of terminating the interaction with a certain partner after a bad experience (a form of partner fidelity) does indeed promote cooperative behaviour. The same principle applies in a continuous snowdrift game (McNamara *et al.* 2008), provided that the variation in cooperativeness is sufficiently large and the same partners can interact with each other sufficiently often. For an N-person prisoner's dilemma game played in a patch-structured population, Ichinose and Arita (2008) showed that a conditional dispersal strategy does not always lead to a more favorable outcome than unconditional dispersal. However, the dispersal strategy yielding the highest fitness was a conditional strategy, and this strategy induced the evolution of cooperation. This matched earlier results by Pepper & Smuts (2002), who showed in a non-evolutionary assortment model that the option to leave non-cooperators leads to positive assortment between cooperators. The joint evolution of partner choice and cooperation tendency was investigated by Hruschka and Henrich (2006), again in the context of an iterated prisoner's dilemma. In this model, already a low level of choosiness allowed the evolutionary emergence and spread of cooperative strategies. Partner choice and partner fidelity are also at center stage in social network theory (Perc & Szolnoki 2010), which does not only consider strategic behaviour but also strategic changes in the structure of the interaction network, such as breaking links with

uncooperative individuals or seeking to establish links with cooperative ones. Various studies (*e.g.* Ohtsuki *et al.* 2006; Fu *et al.* 2008; Zhang *et al.* 2012) arrive at the conclusion that 'rewiring' the existing interaction network can strongly promote and stabilize cooperation.

The studies cited above are important first steps, but they also have several limitations. First, most of these studies are based on game situations where the players repeatedly interact and know each other's identity. In many biological systems, interactions are with anonymous or changing partners, and reliable information on the cooperativeness of these partners is often not available. Second, the strategic structure of the game models studied is often very simple. Many models restrict attention to binary choices (between cooperation and defect) while in natural systems there are often many 'degrees' of cooperation. In other words, cooperativeness is typically a continuous (rather than a discrete or even binary) variable. Third, most cooperation models equate the payoff of a cooperation game (*e.g.*, a prisoner's dilemma game or a public goods game) with fitness. This can be misleading, since the ecological context (*e.g.*, density regulation) and the patterning of the life cycle can strongly affect the evolutionary outcome, both qualitatively and quantitatively (Mylius & Diekmann 1995; Pen & Weissing 2000). Fourth, and most importantly, partner choice and partner fidelity are either implemented by fixed, non-evolvable rules or as evolvable strategies with limited flexibility. In particular, most models assume *a priori* that individuals should have the unidirectional tendency to seek cooperative neighbourhoods and to avoid non-cooperative environments. As we will demonstrate, an opposite tendency can also make perfect sense.

Here, we consider a model for the joint evolution of conditional dispersal and anonymized cooperation that allows for both partner choice and partner fidelity. We consider a species that lives in patches of fixed size where local inhabitants play a public goods game. Individuals may condition their dispersal decisions on the mean level of cooperativeness in their patch, which may result in partner fidelity. Likewise, we allow for conditional settlement where dispersing individuals make their decision on whether to settle dependent on the mean level of cooperativeness of a target patch, which is equivalent to active partner choice.

By means of individual-based evolutionary simulations, we investigate the joint evolution of cooperativeness, conditional dispersal, and conditional settlement, focusing on the question whether such conditional strategies will emerge and subsequently allow the stable establishment of sizable levels of cooperation. No *a priori* assumptions are made with respect to the kind of conditional strategies that could evolve. We also evaluate which kinds of dispersal and settlement strategies are more favourable for the evolution of cooperation and whether different strategies can stably coexist.

THE MODEL

Overview

We consider a patch structured population, in which individuals locally interact in a public goods game and an individual's contribution to the patch-specific public good is a heritable

trait. Contributing to the public good ('cooperation') is costly for the actor and beneficial for all individuals inhabiting a patch. The benefits accrued on a patch owing to individual contributions of (some) inhabitants are equally distributed over all individuals in the patch. Hence, all individuals profit from a high degree of cooperation, while only the contributors pay a cost. The individual cost of public good investment implies that cooperation will be selected against in well-mixed populations.

Generations in our model are discrete and non-overlapping, with a complete life cycle consisting of three phases: a social interaction phase, a dispersal and settlement phase and a reproduction phase. In the social interaction phase, the public goods game is played. Every individual can make a costly contribution to a common good that is shared equally among all patch inhabitants, regardless of their individual contribution. The net effect of contributing and receiving determines an individual's 'condition', which in turn affects its future fecundity in the patch where it will reproduce. During the dispersal and settlement phase, individuals may redistribute over the patches. Each individual has a certain tendency to leave its natal patch and settle in a new patch. A candidate target patch is selected at random but may be rejected if it does not meet the individual's patch quality criteria of cooperativeness. Several rounds of such inspection of patches and rejection may occur before finally settling. Both dispersal and rejection carry a small mortality cost. Dispersal and settlement strategies can be either fixed or conditional on the local productivity of the natal patch and its alternatives after settlement. The reproduction phase starts after all dispersing individuals have settled in a new patch. In each patch, all individuals (both locals and immigrants) produce offspring in proportion to their condition, as determined by the costs and benefits experienced in the social interaction phase. After reproduction, the parents die and the offspring compete for a fixed number of N positions in their natal patch. The N successful individuals then enter the interaction phase, while the remaining individuals die (are removed from the population).

An individual's cooperativeness (*i.e.*, its contribution to the public goods game) and its tendencies to disperse and settle are heritable strategies that are transmitted from parents to offspring, subject to occasional mutations, which allows these strategies to evolve jointly over the generations. We are particularly interested in the evolution of cooperation under four dispersal and settlement scenarios: (1) dispersal and settlement rates are both constant (*i.e.*, not conditional on local cooperativeness) and the same for all individuals (*i.e.*, no heritable variation); (2) the dispersal decision is based on an evolvable conditional strategy; (3) the settlement rule is based on an evolvable conditional strategy; (4) both dispersal and settlement are heritable conditional strategies. We used these scenarios to evaluate whether and under what circumstances conditional dispersal and settlement strategies will favour the evolution of cooperation.

Social interactions

Each patch member i in a given patch contributes to the public good according to its genetically determined investment level x_i. The costs of this contribution are cx_i, where the cost c per unit of investment is fixed parameter. The total investment made in the patch, $x_{tot} = \sum x_k$, generates a revenue bx_{tot} that is distributed over all patch members, irrespective of their contribution to the public good. The benefit b, *i.e.*, the revenue per unit of

investment, is again a fixed parameter. Each of the N patch members receives a revenue $\frac{1}{N}bx_{tot} = b\bar{x}$, where $\bar{x} = \frac{1}{N}\sum x_k$ is the average 'cooperativeness' (= contribution to the public good) in the patch. The difference between benefits ($b\bar{x}$) and costs (cx_i) determine the fecundity of focal individual i, which is given by:

$$W_i = W_0 + b\bar{x} - cx_i \,. \tag{6.1}$$

Here W_0 denotes the baseline fecundity, which is set at a sufficiently high value to prevent fecundities becoming negative. In our model, only relative fecundities (relative to other patch members) are important, so that we can normalize fecundities by dividing all terms in equation (6.1) by b:

$$w_i = w_0 + \bar{x} - (c/b)x_i \,, \tag{6.2}$$

where $w_i = W_i/b$ and $w_0 = W_0/b$. Later on we will systematically vary the cost-benefit ratio c/b. The normalized baseline fecundity w_0 determines the strength of selection: if w_0 is large, the costs and benefits in equation (6.2) have only a small effect on w_i and all individuals have a fairly similar fecundity. A high value of w_0 thus corresponds to weak selection. In the main text, we use $w_0 = 5$. In the appendix (Figure 6.A3), we will also consider the case $w_0 = 2$, corresponding to stronger selection. It is useful to rewrite equation (2) as:

$$w_i = w_0 + \tfrac{1}{N}\sum_{k \neq i} x_k + \left(\tfrac{1}{N} - \tfrac{c}{b}\right)x_i \,. \tag{6.3}$$

This equation reveals that for a low cost-benefit ratio ($\frac{c}{b} < \frac{1}{N}$) the focal individual's investment x_i has also a *positive* net effect on i's fecundity (*i.e.*, w_i increases with x_i). Therefore, the public goods game is a social dilemma only for $\frac{c}{b} > \frac{1}{N}$ when, all other things being equal, individuals are better off by not cooperating but reaping the benefits accumulated by the cooperation of others. In all simulations shown here $N = 50$, implying that the cost-benefit ratio $\frac{c}{b} = \frac{1}{50} = 0.02$ is the borderline case beyond which the public goods game turns into a social dilemma.

Dispersal and settlement

In the dispersal and settlement phase, each individual has the opportunity to leave its natal patch. Depending on the dispersal scenario considered, the dispersal probability d is either constant ($d = d_0$ for all individuals; following Taylor (1988), we chose $d_0 = 0.08$) or an evolvable conditional strategy. In the latter case, the dispersal probability of a given individual depends on the average cooperativeness $\bar{x}$ in the individual's natal patch:

$$d(\bar{x}) = \frac{1}{1 + \exp(-\beta_d \cdot (\bar{x} - \alpha_d))} \,. \tag{6.4}$$

Here, α_d and β_d are heritable parameters describing how the individual will respond to the average public goods investment $\bar{x}$ encountered in its patch. As illustrated in Figure 6.1, equation (6.4) represents a logistic function that can either increase (when $\beta_d > 0$; 'type 1') or decrease ($\beta_d < 0$; 'type 2') with $\bar{x}$. This function has a turning point in $\bar{x} = \alpha_d$, at which

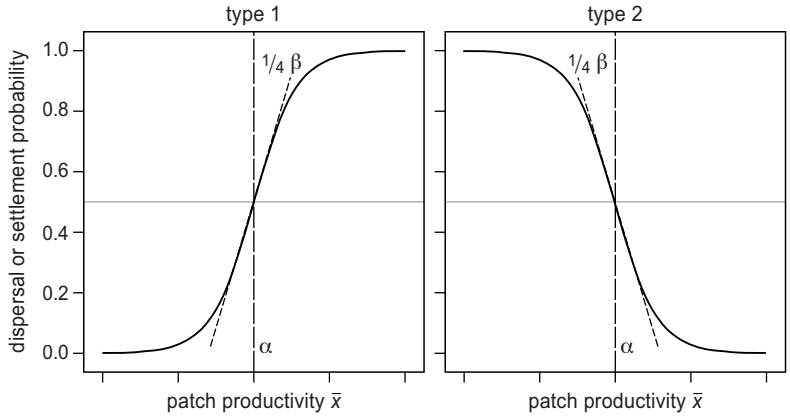


Figure 6.1 Logistic functions describing a conditional dispersal or settlement strategy. Two examples of a logistic function $f(\bar{x}) = [1 + \exp(-\beta(\bar{x}-\alpha))]^{-1}$ describing the tendency for a certain behaviour (dispersal or settlement) in relation to the productivity $\bar{x}$ of a patch. In our model patch productivity corresponds to patch cooperativeness. The two (heritable) parameters α and β characterizing the function have a clear interpretation: $f(\bar{x}) = \frac{1}{2}$ for $\bar{x} = \alpha$, and $f'(\bar{x}) = \frac{1}{4}\beta$ at $\bar{x} = \alpha$. Logistic functions of (a) type 1 ($\beta > 0$) and (b) type 2 ($\beta < 0$) could both evolve in our model.

$d(\bar{x}) = d(\alpha_d) = \frac{1}{2}$. The parameter β_d is proportional to the slope of the logistic function at the turning point ($d'(\alpha_d) = \frac{1}{4}\beta_d$). If an individual has a positive β_d (type 1), it has the tendency to leave productive patches (large values of $\bar{x}$), while individuals with a negative β_d (type 2) tend to leave patches with low productivity. When β_d is large in absolute value, the results is an all-or-nothing dispersal strategy, of either always leaving or always staying, depending on whether $\bar{x}$ is larger or smaller than α_d. Dispersal is, however, costly so that compared to individuals staying in their natal patch, the viability of dispersing individuals is reduced by a factor $1 - m_d$ with $m_d = 0.1$ in our simulations.

Dispersing individuals encounter patches at random. Depending on the settlement scenario considered, they either settle on the first patch encountered or they base their settlement decision on an evolvable conditional strategy. In the latter case, the probability to settle in a candidate target patch depends on the average cooperativeness $\bar{x}$ in that patch:

$$d(\bar{x}) = \frac{1}{1 + \exp(-\beta_s \cdot (\bar{x} - \alpha_s))} \ . \qquad (6.5)$$

As with dispersal, also the settlement strategy (equation (6.5)) is characterized by two heritable parameters α_s and β_s. An individual can reject several patches; while it explores new patches offered until a target patch is eventually accepted. However, rejecting a patch is costly in that it reduces the survival by a factor $1 - m_s$. In our simulations, the rejection-related mortality was $m_s = 0.01$.

Reproduction and inheritance

After all individuals have settled, the members of each patch produce offspring in proportion to the fecundity accrued in their natal patch (equation (6.2)). Of the offspring

produced in each patch, N are chosen at random to form the patch members entering the social interaction phase in the next generation, while parents and all other offspring die.

Individuals are haploid and reproduce clonally, so that each individual is genetically characterized by its alleles $(x,\alpha_d,\beta_d,\alpha_s,\beta_s)$ at five jointly evolving loci. Allelic values are real numbers that determine the individual's cooperativeness (*i.e.*, its contribution to the public good) and its condition dependent dispersal and settlement strategy. In the dispersal scenarios where the dispersal and/or settlement decision is not evolvable and based on a fixed probability the corresponding genetic parameters $[(\alpha_d,\beta_d)$ and/or $(\alpha_s,\beta_s)]$ have no effect and evolve by genetic drift. When reproducing, parents transmit all five alleles to their offspring, but each locus mutates with probability μ. In such cases, a small number drawn from the uniform distribution on the interval $(-\delta,\delta)$ is added to the allele at the corresponding locus. To prevent a mutation bias at zero, allelic values at the cooperativeness locus were allowed to become negative (a negative value of x induces a contribution of zero to the public goods game). However, mutations shifting x below –0.1 were not allowed, in order to prevent x drifting to very negative values (from where the subsequent evolution of cooperativeness would be very difficult to achieve). In our simulations, we used a per-locus mutation rate $\mu = 0.01$ and a maximal mutational step size $\delta = 0.1$.

Simulation settings

We simulated populations consisting of 50,000 individuals that where distributed over 1,000 patches, with each patch harbouring $N = 50$ individuals. In the simulations reported in the main text all individuals were initialized at the allelic values $x = \alpha_d = \alpha_s = 0.5$ and $\beta_d = \beta_s = 0$. To check for the robustness of our conclusions, we also initialized β_d and/or β_s at positive (type 1 logistic function) or negative (type 2 logistic function) values, which produced results given in the appendix (Figure 6.A2).

For all four dispersal and settlement scenarios we considered six different cost-benefit ratios ($c/b = 0.0001$, 0.001, 0.01, 0.02, 0.06, 0.1), including two cases where the public goods game corresponds to a social dilemma ($c/b > 0.02$). The figures in the main text report on the cost-benefit values $c/b = 0.06$ (social dilemma) and $c/b = 0.01$ (no social dilemma); the results of the other cost-benefit ratios are given in the appendix.

For each dispersal and settlement scenario and each parameter combination considered, we ran 100 replicate simulations. Most simulations were run for 50,000 generations, but we regularly ran longer simulations (>200,000 generations) to check for the robustness of our results.

The simulation program was coded in C^{++} (code available upon request from A.I.), and compiled in g^{++} (4.3.4; Free Software Foundation, Inc. 2008).

RESULTS

Evolution of cooperation when the costs of cooperation are low

Let us first consider a small cost-benefit ratio ($c/b = 0.01$), where the benefits of contributing to the public good exceed the costs of the contribution and the public goods game is no social dilemma. Figure 6.2 shows five representative simulation runs for each of the

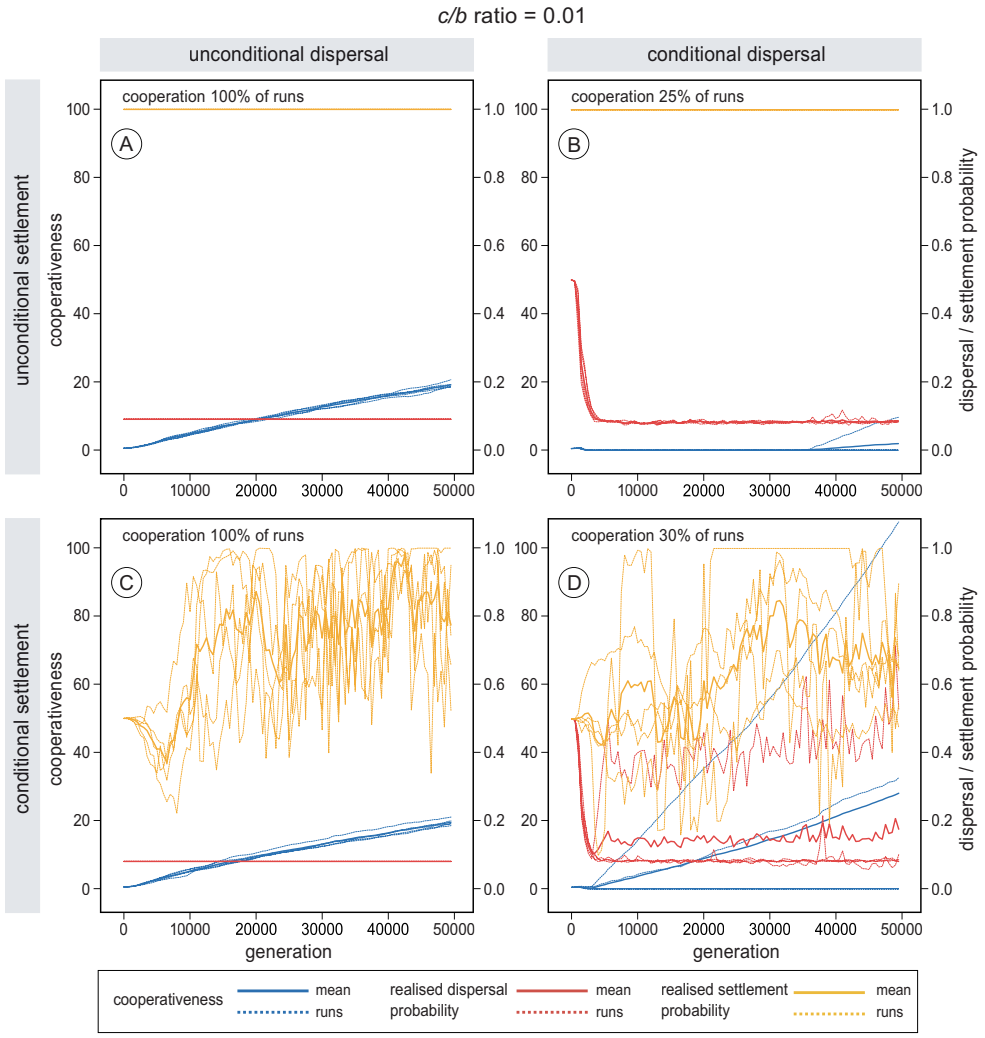


Figure 6.2 Evolution of cooperation when the public goods game is no social dilemma. Evolution of cooperativeness (blue), mean dispersal rate (red) and mean settlement rate (orange) in case of a low cost-benefit ratio ($c/b = 0.01$) under four scenarios: (A) fixed dispersal probability ($d = 0.08$) and fixed settlement probability ($s = 1.0$); (B) evolvable conditional dispersal strategy and fixed settlement probability; (C) fixed dispersal probability and evolvable conditional settlement strategy; (D) evolvable conditional dispersal and settlement strategies. Each plot shows 5 randomly selected replicate simulation runs (dashed lines) and their means (bold lines). Left y-axis: cooperativeness, right y-axis: probability of dispersal or settlement. The figures in the top left corner of each plot give the percentage of 100 simulation runs resulting in the evolution of cooperation.

four dispersal and settlement scenarios. When the dispersal probability was fixed (at $d =$ o.o8) and unconditional on local productivity, cooperativeness steadily increased over the generations, reaching a level of about within 50,000 generations (Figure 6.2AC). This

happened in all 200 simulation runs for these two scenarios. Surprisingly, however, the joint evolution of a more flexible conditional dispersal strategy inhibited cooperativeness and often led to the breakdown of cooperation (Figure 6.2BD). Irrespective of the settlement scenario, all 200 simulation runs showed an all-or-nothing pattern: while some runs resulted in consistently increasing high levels of cooperativeness (sometimes reaching a level of $x > 100$ within 50,000 generations), cooperativeness converged to zero in the majority of runs. In fact, cooperation only got off the ground in 25% of the 100 runs with fixed settlement and 30% of the runs with evolvable conditional settlement. This small difference in simulation outcomes is not significant ($P = 0.431$, Fisher exact test); apparently there is no synergistic effect of conditional dispersal and conditional settlement on the evolution of cooperation under low cost-benefit ratios.

As shown in the appendix, the same outcome was observed for even lower cost-benefit ratios (Figure 6.A1). In case of fixed, unconditional dispersal, cooperativeness always evolved, and the level reached within 50,000 generations was negatively related to the cost-benefit ratio. In case of the joint evolution of conditional dispersal, the simulations exhibited an all-or-nothing pattern, where the percentage of simulation runs resulting in cooperation was lowest for the lowest cost-benefit ratios.

Evolution of cooperation in a social dilemma

When the cost-benefit ratio is larger than the public goods game becomes a social dilemma. For $c/b = 0.06$, Figure 6.3 shows five representative simulation runs for each of the four dispersal and settlement scenarios. When the dispersal probability was fixed and unconditional on local productivity, cooperation did not get off the ground, irrespective of the settlement scenario (Figure 6.3AC). In fact, cooperativeness rapidly converged to zero in all 200 simulation runs with fixed and unconditional dispersal. In contrast, high levels of cooperativeness could evolve when conditional dispersal could jointly evolve (Figure 6.3BD). As in the case of a low cost-benefit ratio, all 200 simulations were of an all-or-nothing type: either cooperativeness dropped to zero or it rapidly evolved to ever increasing high levels (typically $x > 100$ within 50,000 generations). In case of $c/b = 0.06$, 66% of the 100 runs with fixed settlement and 63% of the runs with evolvable conditional settlement resulted in the evolution of cooperation. This small difference in simulation outcomes is not significant ($P = 0.767$, Fisher's exact test), so there is again no evidence for a synergistic effect of conditional dispersal and conditional settlement on the evolution of cooperation. Both percentages are significantly higher than the corresponding figures (25% and 30%, respectively) that were obtained for $c/b = 0.01$ in Figure 6.2 ($P < 0.001$, Fisher exact test).

As shown in the appendix, the same simulation outcome was observed for a higher cost-benefit ratio ($c/b = 0.1$; see Figure 6.A1). Cooperativeness always converged to zero in case of fixed dispersal, and to an all-or-nothing pattern when cooperativeness jointly evolved with conditional dispersal. Although the cost-benefit ratio was higher than in the simulations of Figure 6.3, cooperation evolved in an even higher percentage of runs (92% with fixed settlement and 90% with evolvable conditional settlement).

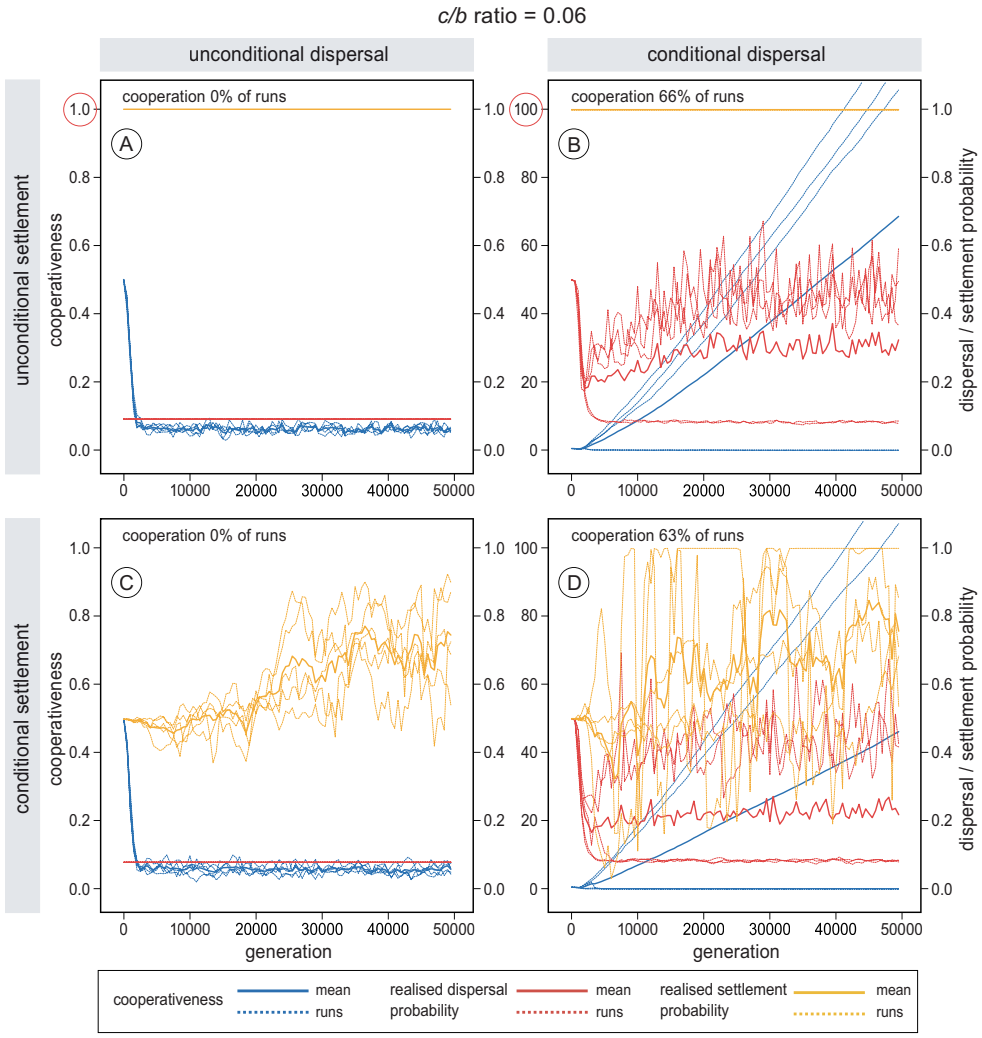


Figure 6.3 Evolution of cooperation in case of a social dilemma. Evolution of cooperativeness (blue), mean dispersal rate (red) and mean settlement rate (orange) when the public goods game is a social dilemma ($c/b = 0.06$). For the same dispersal and settlement scenarios as in Figure 6.2, each plot shows 5 randomly selected replicate simulation runs (dashed lines) and their means (bold lines). Left y-axis: cooperativeness (ranging from 0 to 1 in (A) and (C) and from 0 to 100 in (B) and (D)); right axis: probability of dispersal or settlement. The figures in the top left corner of each plot give the percentage of 100 simulation runs resulting in the evolution of cooperation.

A closer look at the evolution of conditional dispersal

From the results until now, we can conclude that conditional dispersal is a potent factor that can both promote and hinder the evolution of cooperation. Moreover, the joint evolution of cooperativeness and conditional dispersal may lead to either high levels of cooperativeness or the disappearance of cooperation. Figure 6.3 shows that in the cooperative

runs, the average dispersal rate and the average settlement rate converged to relatively high values ($d \approx 0.5$; s often attaining the maximal value of 1.0), while the values obtained in the non-cooperative runs were much lower ($d \approx 0.05$, $s \approx 0.5$) (Figure 6.3BD). But what determines whether a simulation run will be 'cooperative'? To answer this question, we investigated two specific simulation runs (for the social dilemma case $c/b = 0.06$) in more detail (Figure 6.4). One of these runs (upper panels of the figure) resulted in high levels of cooperation, while cooperativeness converged to zero in the other run (lower panels). The replicated simulations in Figure 6.4 are typical in that cooperativeness first declined from the initial value $x = 0{,}5$ and subsequently sharply increased again as can be clearly seen in Fig 6.4E. While x continued to increase in the cooperative runs, it abruptly declined and converged to zero in the non-cooperative runs. Figure 6.4 also provides a more detailed view of how the conditional dispersal strategy jointly evolved with the tendency to cooperate. In the cooperative run, the parameter α_d of the dispersal strategy (equation (6.4)) closely matched the average cooperativeness $\bar{x}$ in the population, while the parameter β_d evolved to rather large positive values. As a consequence, the dispersal strategy corresponds to a type-1 logistic function that resembles a 'bang-bang' strategy (Figure 6.4D): individuals using this strategy leave their patch, when the patch productivity is larger than α_d, and they stay on their patch when $\bar{x} < \alpha_d$. In non-cooperative runs (Figure 4EFGH), both types of logistic function ($\beta_d > 0$ and $\beta_d < 0$) coexist in the same population for extended periods of time. Initially, type-1 strategies are as predominant as in the cooperative runs. Eventually, however, they get outcompeted by a type-2 strategy, where individuals tend to leave unproductive patches while they tend to stay in cooperative patches. These outcomes are opposite to what one might have expected: the standard pattern associated with partner fidelity (*i.e.*, leaving unproductive patches and staying in cooperative environments) leads to the decay of cooperation, while the opposite pattern allows for the evolution of cooperation even in case of large cost-benefit ratios.

Robustness of the results

The simulations reported above were initialized at $\beta_d = 0$, that is, dispersal was initially not condition dependent. It is conceivable that starting with a positive or negative value of β_d might lead to a different outcome, since it might give the evolution of cooperation a head start. We therefore initialized some simulations with the values $\beta_d = +0.1$ and $\beta_d = -0.1$, where the associated initial values of α_d were chosen such that $d(x_{init}) = d(0.5) = 0.15$ for the logistic function in equation (6.4). In line with our earlier results, cooperation never evolved when the population was initialized with a type-2 dispersal strategy ($\beta_d = -0.1$). Cooperation did evolve in about 50% of the simulations when the population was initialized at $\beta_d = +0.1$, but it got off the ground less easily than in our standard set-up. Figure 6.A2 in the appendix shows a typical example. Cooperativeness did not evolve for an extended period of time (about 30,000 generations). In this period, α_d declined steadily from its initial value $\alpha_d = 17.85$. Once it had reached a sufficiently small value, both β_d and cooperativeness started to increase. In sum, cooperation indeed evolves frequently when β_d values are initialized positive, whereas it does not evolve when β_d values are initialized negative.

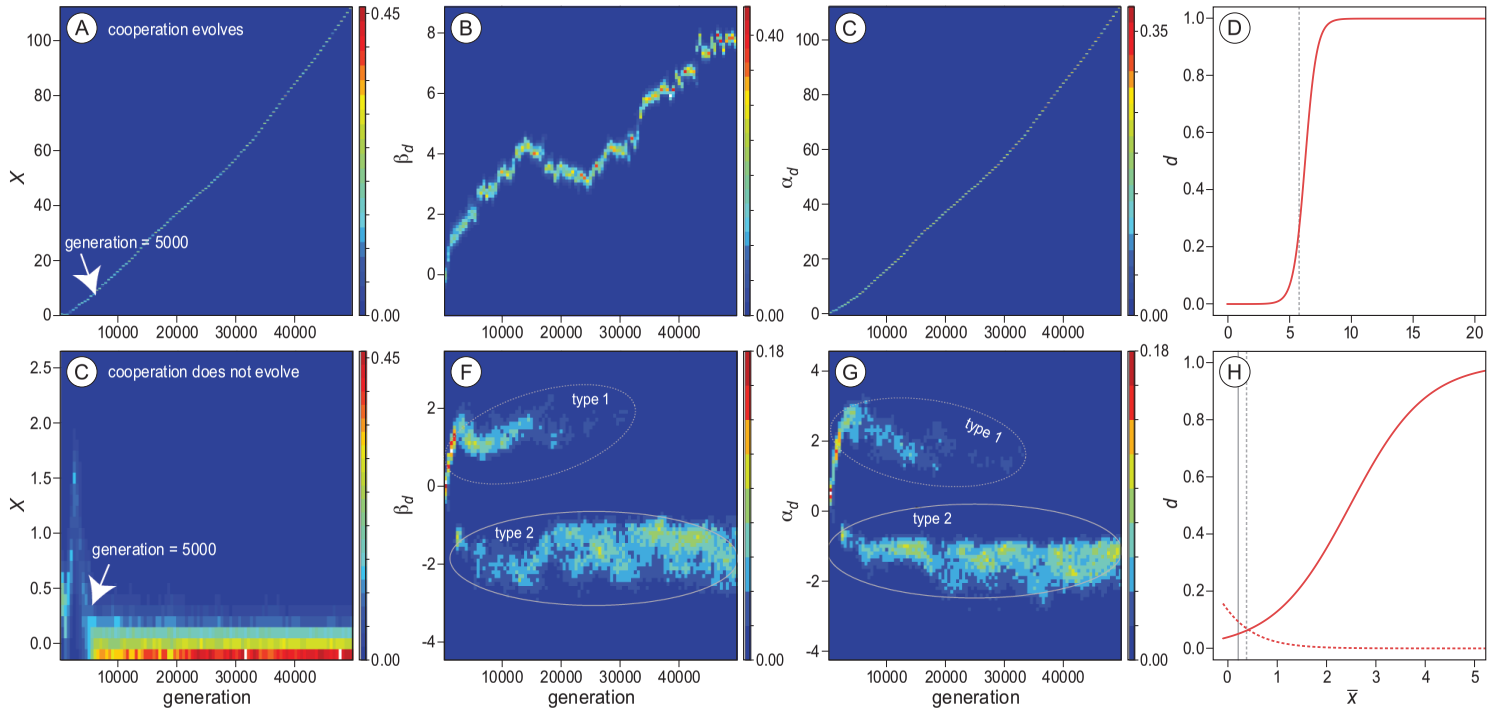


Figure 6.4 Joint evolution of cooperativeness and conditional dispersal in replicate simulation runs leading to either the evolution of cooperation (top panels) or the disappearance of cooperation (bottom panels). The six panels to the left are heat plots illustrating the evolution of the frequency distribution of the alleles for cooperativeness x (A, C) and the parameters β_d (B, F) and α_d (C, G) characterizing the conditional dispersal strategy. For generation 5,000, panels (D) and (H) show representative dispersal strategies and the average cooperativeness of those individuals expressing these strategies (vertical lines). In the cooperative simulation run (top panels), a single type-1 dispersal strategy is predominant. In the non-cooperative simulation run (bottom panels), two different dispersal strategies stably coexist for extensive periods of time. In panel (H), the type-1 strategy and the cooperativeness of type-1 strategists is indicated by solid lines; the type-2 strategy and the corresponding cooperativeness is indicated by a dashed line.

To further investigate the robustness of our results, we increased the strength of selection by reducing baseline fecundity from its standard value 5 to the lower value 2. Figure 6.A3 in the appendix shows the results for the two cost-benefit ratios $c/b = 0.01$ (no social dilemma) and $c/b = 0.06$ (social dilemma). Qualitatively, our earlier results were recovered, but they were quantitatively different in showing that stronger selection accelerates (as expected) the enhancement of cooperation when dispersal becomes conditional and cost-benefit ratios are high, whereas it inhibits cooperation more effectively when cost-benefit ratios are low.

DISCUSSION

It has repeatedly been argued that partner choice and partner fidelity may strongly promote the evolution of cooperation (Bull & Rice 1991; Sachs *et al.* 2004; Foster & Wenseleers 2006). Cooperation models including partner choice and partner fidelity (Sherratt & Roberts 1998; Hruschka & Henrich 2006; Ichinose & Arita 2008; McNamara *et al.* 2008; Zhang *et al.* 2012) do indeed conclude that cooperation can evolve if individuals tend to leave uncooperative environments and tend to settle in cooperative neighbourhoods. However, all these models implemented *a priori* a fixed direction of the partner choice and/or a partner fidelity mechanism: individuals were assumed to have the tendency to move away from non-cooperative environments and towards cooperative environments. To our knowledge, the present model is the first to consider the evolutionary emergence of such directionality as well as the joint evolution of cooperativeness, partner choice and partner fidelity.

Our results confirm that conditional dispersal (a mechanism related to partner fidelity) has an important effect on the evolution of cooperation, but that conditional settlement (a mechanism related to partner choice) has only a marginal effect. Interestingly, the effect of conditional dispersal on cooperativeness is not universally positive. In those cases where cooperation seems to be the standard option (a low cost-benefit ratio making cooperation individually advantageous), conditional dispersal often led to the breakdown of cooperation. Conditional dispersal did have a positive effect on cooperativeness in case of a social dilemma situation, but also this effect manifested itself in an unexpected way. Whenever a population evolved the expected pattern associated with partner fidelity (*i.e.*, to leave non-cooperative environments), cooperativeness did *not* evolve. In contrast, cooperativeness evolved in those cases where individuals tended to leave patches with a high degree of cooperation.

How can these patterns be explained? Presumably, two features of our model are responsible for this: local population regulation and the timing of dispersal in relation to other life-history events. The local population was reduced to N individuals at the start of each generation. In other words, we did not consider the possibility of local resource enhancement, that is, a positive effect of local cooperativeness (= productivity) on the local carrying capacity, also known as 'population elasticity' (Queller 1994; Le Galliard *et al.* 2003; Janssen & Goldstone 2006). As a consequence, if individuals tend to be philopatric a high degree of local cooperation automatically leads to a high degree of local

competition, since more offspring will have to compete for the N positions if local fecundity is high. Our model also assumes that social interactions precede dispersal and settlement. As a consequence, individuals can evade local competition by leaving patches with high productivity. In view of these considerations, a tendency to leave cooperative patches makes perfect sense, because an individual can only really profit from the benefits of cooperation if it goes away, thereby avoiding the severe competition otherwise experienced by its offspring. It has long been known that enhanced local competition may cancel the benefits of local cooperation (Queller 1992; Taylor 1992; West *et al.* 2002). The evolved dispersal strategies in our simulations annihilate this effect by avoiding competition in a productive environment.

Why does 'inverse partner fidelity' (leaving productive environments) favour the evolution of cooperation in a social dilemma situation? Once a type-1 dispersal strategy has become established, it is understandable how cooperativeness can evolve. Within a patch, cheaters (*i.e.*, individuals with an atypically low degree of cooperation) can exploit the cooperativeness of other patch members, but they also lower the average productivity of their patch. As a consequence, cheaters (and other patch members) will tend to stay, while newcomers to the patch will predominantly come from patches of high productivity. Both factors enhance the local competition experienced by the offspring of cheaters, and this effect is apparently sufficiently strong to overcompensate for the immediate benefits of cheating. The lesson is that the payoffs (equation (6.1)) in the public goods game are not necessarily reliable proxies of individual fitness, as they correspond to one fitness component (fecundity), while total fitness also depends on other factors, such as the intensity of local competition. Once 'inverse partner fidelity' is established in the population, it becomes clear why conditional settlement decisions ('partner choice') only had a marginal effect on the evolution of cooperation. In our model, settlement could evolve to be based on the past productivity of a patch. If individuals have the tendency to leave productive patches, past cooperativeness becomes an unreliable predictor of future cooperativeness. In addition, the evolved value of α_d closely matched the mean cooperativeness $\bar{x}$ in the population (Figure 6.4AC). Hence, individuals with a type-1 dispersal strategy tend to leave patches of above-average cooperativeness and, as a consequence, they will on average encounter patches of a lower cooperativeness than in their natal patch. Accordingly, individuals can better avoid the costs of choosiness at settlement and settle on the first patch they encounter.

Why does conditional dispersal hamper the evolution of cooperation in case of low cost-benefit ratios that do not constitute a social dilemma? In fact, the same kind of reasoning applies as in the social dilemma situation. When $c/b < 1/N$, an individual extracts more benefits from its investment than the costs of this investment. However, the other patch members will profit even more from this investment (since they do not have to pay the costs). When staying in a cooperative patch, cooperative individuals therefore have to face competition with other philopatric individuals whom they in fact helped to become strong competitors. Thus, also in case of a very low cost-benefit ratio individuals profit from avoiding competition by leaving cooperative patches. If, on the other hand, a type-2 strategy gets the upper hand in the early stages of evolution, cooperation does not persist since its benefits are nullified by the costs of local competition described above.

CONCLUSIONS

Our analysis shows that the joint evolution of cooperativeness and conditional dispersal has features that – at least at first sight – are counter-intuitive. In the model considered, conditional dispersal could hamper the evolution of cooperation, and when it favoured the evolution of cooperation, the dispersal strategy had an unexpected shape. We do not pretend that exactly the same results will apply to all cooperation models. In fact, the evolutionary outcome would presumably have been different if the causal link between local productivity and the intensity of local competition would have been broken (or weakened) by local resource enhancement, if dispersal and settlement would have preceded the local interaction phase; or if individuals could have based their settlement decisions on indicators of future (rather than past) cooperativeness. Nevertheless, a few general conclusions can be drawn from our study. First, the evolution of conditional strategies is often difficult to predict *a priori*. This insight is a cornerstone of classical game theory (Van Damme 1991; Rasmusen 2006), but it is not yet firmly established in evolutionary ecology. Yet, also in biology a variety of models have arrived at the conclusion that the evolution of conditional strategies may have surprising outcomes ([*e.g.*, the evolution of winner-loser effects in the absence of any difference in resource-holding potential, Van Doorn *et al.* 2003; the strategic disadvantage of being the informed party in a conflict of interest, Pen & Taylor 2005; the erosion of sexual selection by conditional sex allocation decisions, Fawcett *et al.* 2011]. Second, conditional strategies often coexist for extended periods of time (as the type-1 and type-2 dispersal strategies in our model; see Figure 6.4FG). Again, there is a variety of examples for this, including the coexistence of communication strategies (Botero *et al.* 2010) and the coexistence of foraging strategies (Giske *et al.* in press). Third, the outcome of evolution may strongly depend on the order of life history events and ecological details (see also Mylius & Diekmann 1995; Pen & Weissing 2000). Since the payoffs of a cooperation game (like the public goods game in our model) are closely related to fitness, they are often viewed as proxies for fitness. As we have seen in our model, this may be a problematic inference, since fitness also depends on factors like local competition, which in turn reflect population regulation and the timing of life history events such as dispersal.

Acknowledgements

We thank Yiming Yu, Joke Bakker and Marten van de Sanden for help with the development and implementation of the model. Support for this work was obtained through a PhD fellowship from the Centre for Ecological and Evolutionary Studies, University of Groningen and by grants from the Langerhuizen Fund and Nicolaas Mulerius Fund, awarded to ABFI. The Centre for High-Performance Computing and Visualisation at the University of Groningen provided access to the Millipede High Performance Cluster.

APPENDIX

This appendix contains additional simulation results that illustrate the robustness of the results discussed in the main text. The main text focused on two cost-benefit ratios (c/b = 0.01 and 0.06). Figure 6.A1 shows that similar results are obtained for a range of cost-benefit ratios (ranging from 0.001 to 0.1). Figure 6.A2 shows some simulations that were not initialized with $\beta_d = 0$ but with a positive value. Figure 6.A3 considers a lower baseline fecundity than the standard value ($w_0 = 5$) used in the main text. All results presented in this appendix are discussed in the main text.

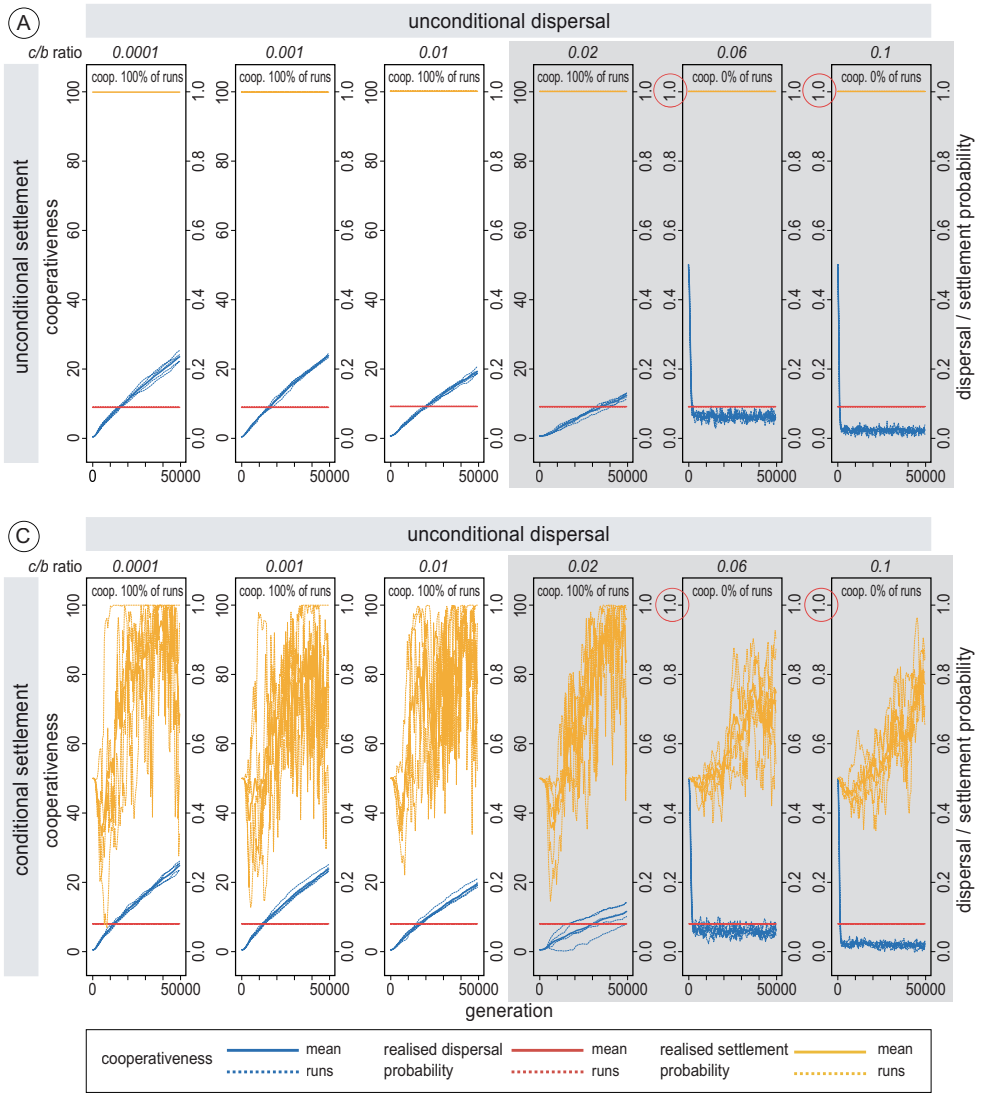


Figure 6.A1 Effect of the cost-benefit ratio on the evolution of cooperation under various dispersal and settlement scenarios. Evolution of cooperativeness (blue), mean dispersal rate (red) and mean settlement rate (orange) for six cost-benefit ratios and four dispersal scenarios: (A) fixed dispersal probability ($d = 0.08$) and fixed settlement probability ($s = 1.0$); (B) evolvable conditional dispersal strategy and fixed settlement probability; (C) fixed dispersal probability and evolvable conditional settlement strategy; (D) evolvable conditional dispersal and settlement strategies. Each plot shows 5 randomly selected replicate simulation runs (dashed lines) and their means (bold lines). Left y-axis: cooperativeness (ranging either from 0 to 100 or from 0 to 1, see highlight); right y-axis: probability of dispersal or settlement The figures in the top left corner of each plot give the percentage of 100 simulation runs resulting in the evolution of cooperation. For each scenario, the three left-most panels ($c/b = 0.0001, 0.001, 0.01$) correspond to a public goods game that is no social dilemma, while the two right-most panels ($c/b = 0.06, 0.1$) refer to social dilemma situations (highlighted with gray background). The fourth panel is the borderline case $c/b = 1/N = 0.02$.

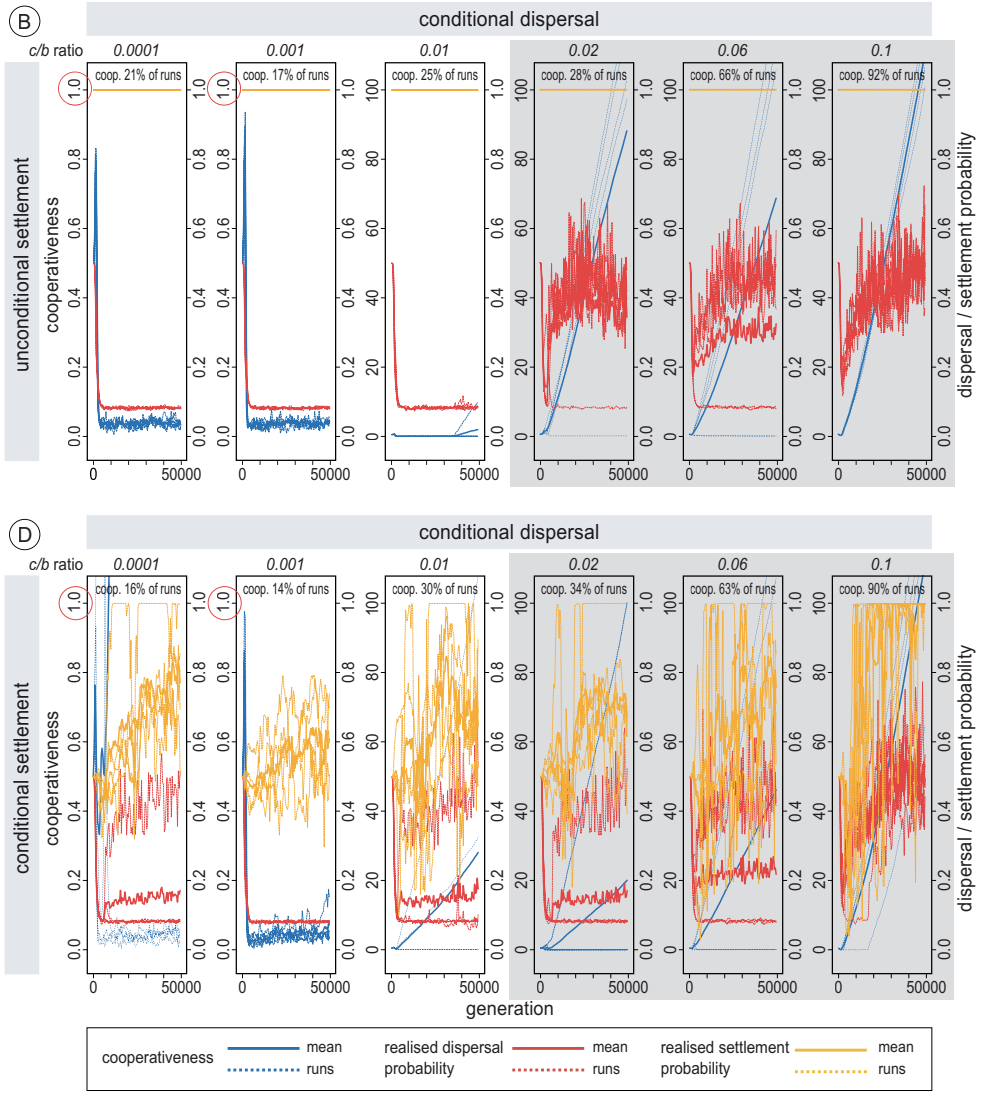


Figure 6.A1 Continued

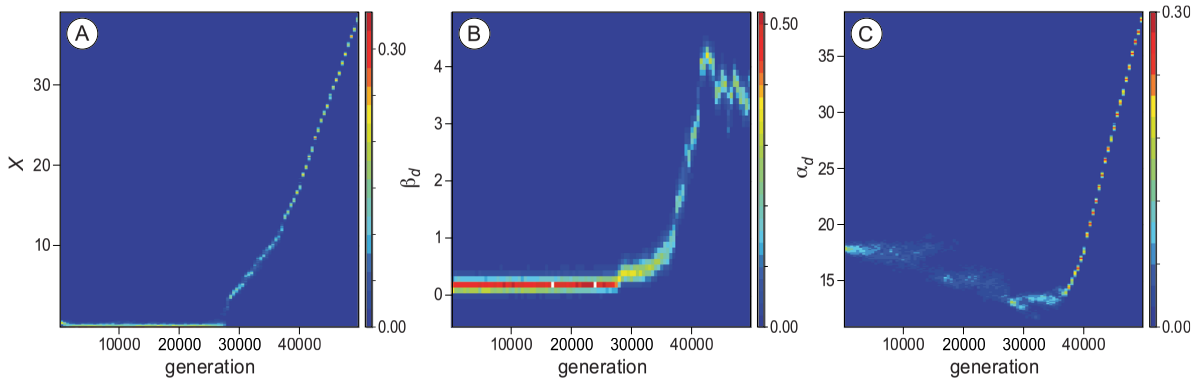


Figure 6.A2 Evolution of cooperation and conditional dispersal when dispersal is initialized as a conditional type-1 strategy. Heat plots corresponding to those in Figure 4ABC illustrating the evolution of the frequency distribution of the alleles for cooperativeness x (A) and the parameters β_d (B) and α_d (C) characterizing dispersal in case of a social dilemma ($c/b = 0.06$). In contrast to the standard initialization used in the main text ($\beta_d = 0$, $\alpha_d = x_{init} = 0.5$), the dispersal parameters were now initialized at $\beta_d = 0.1$, $\alpha_d = 17.85$, which yield an initial dispersal rate of $d(x_{init}) = 0.15$. Although type-1 dispersal was given a head start, it took much longer (than in Figure 6.4ABC) before cooperativeness got off the ground. Simulations initialized with $\beta_d = -0.1$ always resulted in the disappearance of cooperation.

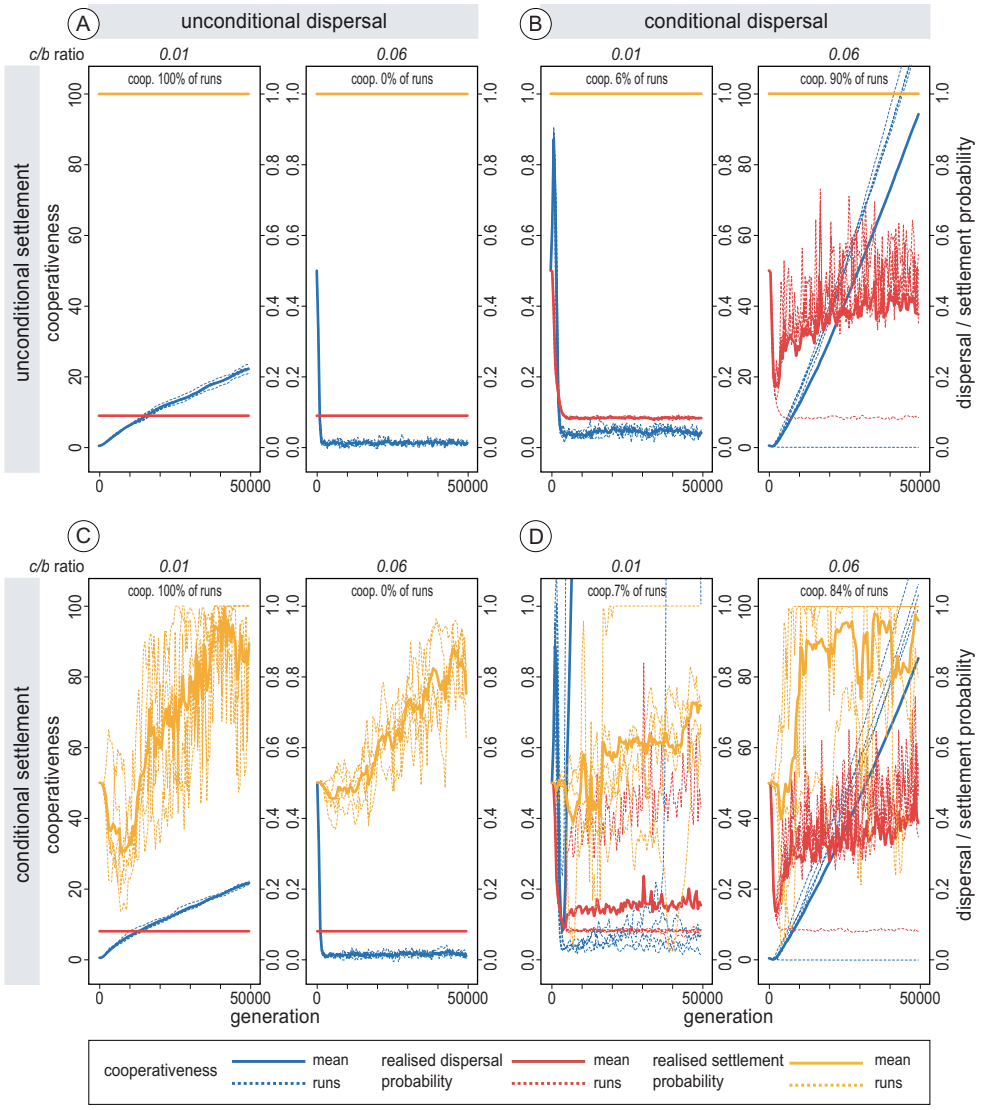


Figure 6.A3 Effect of the strength of selection on the evolution of cooperation. The simulations shown correspond to those of Figure 6.2 ($c/b = 0.01$; no social dilemma) and Figure 6.3 ($c/b = 0.06$; social dilemma) in the main text, but the strength of selection in the social dilemma was increased by reducing basal fecundity from $w_0 = 5$ to $w_0 = 2$. As before, the panels depict the evolution of cooperativeness (blue), mean dispersal rate (red) and mean settlement rate (orange) in four scenarios: (A) fixed dispersal probability ($d = 0.08$) and fixed settlement probability ($s = 1.0$); (B) evolvable conditional dispersal strategy and fixed settlement probability; (C) fixed dispersal probability and evolvable conditional settlement strategy; (D) evolvable conditional dispersal and settlement strategies. Each plot shows 5 randomly selected replicate simulation runs (dashed lines) and their means (bold lines). Left y-axis: cooperativeness (ranging from 0 to 1.0 or from 0 to 100); right y-axis: probability of dispersal or settlement. The figures in the top left corner of each plot give the percentage of 100 simulation runs resulting in the evolution of cooperation.

CHAPTER 7

Does the joint evolution of cooperativeness and conditional dispersal promote the evolution of mutualism?

Aniek B.F. Ivens
Ido Pen
Jacobus J. Boomsma
Franz J. Weissing

ABSTRACT

Mutually beneficial interactions between species are common and of considerable ecological importance, but the evolutionary stability of such relationships is not well understood. This is because costly cooperation can be exploited by cheaters reaping the benefits without offering much, if anything in return. It has been argued that partner choice (actively selecting cooperative interaction partners) and partner fidelity (continuing profitable relationships and abandoning unprofitable ones) might promote the evolution of mutualism. Here we use individual-based simulations to investigate whether and under which circumstances the joint evolution of cooperativeness, partner choice and partner fidelity does indeed result in a mutualistic outcome. Our model considers a patch-structured environment with fixed patch size where individuals of two species interact locally by producing costly commodities that are beneficial for the patch members of the other species. Production rate of the commodity ('cooperativeness'), conditional dispersal strategy ('partner fidelity'), and conditional settlement strategy ('partner choice') are all heritable traits that jointly evolve in both species. To make the results comparable with an earlier model for the evolution of intraspecific cooperation, we focused on a symmetric scenario where species have identical life histories and fitness parameters. We show that interspecific cooperation can evolve, but only under extremely low cost-benefit ratios. When these conditions are met, both conditional dispersal and conditional settlement promote cooperation and have a synergistic effect. The evolved pattern of dispersal is opposite to the common assumption in models of partner fidelity that individuals will stay in cooperative patches and leave uncooperative patches. We explain this finding by the strong local competition in patches where productivity is high. Although the two species considered in our model were identical, evolved patterns of cooperation were typically unilateral, with one species contributing a lot and the other very little. This matches the observation that many natural mutualisms exhibit a strong asymmetry between partners.

INTRODUCTION

Mutualistic cooperation between species is widespread and of major importance to complex life (Leigh 2010). Mutually beneficial interactions range from ancient endosymbionts that evolved into organelles such as mitochondria and plasmids, to pollination networks that help stabilizing ecosystems (Margulis 1971; Stachowicz 2001; Bastolla *et al.* 2009). The emergence and evolutionary stability of mutualisms are, however, not well understood. How can species evolve to invest into costly helping of other species, whereas the benefits could also be reaped for free by exploiting the other species as a parasite (Herre *et al.* 1999; Bronstein 2001; Bergstorm *et al.* 2002; Leigh 2010)? Conditional behaviour is assumed to play a major role in the evolution of interspecific cooperation. The two most important mechanisms of conditional dispersal are partner fidelity, the option to either continue or abandon current interactions and partner choice, the option to only engage in interactions with selected partners (Bull & Rice 1991; Foster & Wenseleers 2006). By inducing positive assortment between cooperators of both interacting species, partner choice and partner fidelity might pave the way for the evolution of cooperation (Nowak & May 1992; Frank 1994; Pepper & Smuts 2002; Foster & Wenseleers 2006).

Several modeling studies (Sherratt & Roberts 1998; Ichinose & Arita 2008; McNamara *et al.* 2008) have shown that conditional dispersal can promote cooperation between individuals of the same species. However, most of these models considered a restricted set of cooperation and/or dispersal strategies. In a previous study (Chapter 6), we considered a more flexible strategic structure. We showed that the joint evolution of cooperativeness and conditional dispersal can both promote (for high cost-benefit ratios) and impede (for low cost-benefit ratios) the evolution of cooperation in a public goods game. Cooperation did only evolve when the (jointly evolving) conditional dispersal strategy was opposite to the pattern included *a priori* in earlier models: individuals tend to leave cooperative patches and to stay in non-cooperative patches. This counter-intuitive result can be explained by the fact that the beneficial effects of local cooperation are annihilated by the intense local competition in cooperative and, hence, productive environments. If local cooperation induces intense local competition, it makes perfect sense to leave cooperative patches. In our model (Chapter 6), the strong link between local cooperation and local competition was caused by the fact that patch sizes were constant. The outcome might have been different in case of local resource enhancement, that is, if local competition would have a positive effect on local carrying capacities. Yet, our study illustrates that the role of partner choice and partner fidelity is less straightforward than has been suggested.

Only a limited number of studies has so far focused on the interplay between dispersal and the evolution of inter-specific cooperation. In a model of pair-wise interactions between two species inhabiting dual lattices it was shown that mutualism can only evolve when dispersal distances are limited (Yamamura *et al.* 2004), a result that was confirmed for joint evolution of cooperativeness and dispersal distance (Mack 2012) and for evolving cost-benefit ratios (Doebeli & Knowlton 1998). In these models, dispersal was unconditional and the results could be explained by positive assortment of cooperators. However, Doebeli & Knowlton (1998) also showed that the likelihood of mutualistic evolution increased when hosts were allowed to take along symbionts that had previously experi-

enced a satisfyingly cooperative interaction. Such conditional association with cooperative partners has also been shown to promote mutualism in an asymmetric model of joint evolution of partner choice in a choosy host and cooperativeness of both hosts and symbionts (Foster & Kokko 2006). In addition, the fitness calculations of Foster & Wenseleers (2006) suggest that mutualism evolves most readily when both species can actively select their partners through partner fidelity and partner choice.

Hitherto, the interplay between dispersal and mutualism has mostly been studied for unconditional dispersal and when dispersal was conditional, individuals were *a priori* assumed move away from non-cooperators and/or towards cooperators. In the model presented here, we explore the joint evolution of conditional dispersal and cooperativeness in two interacting species without *a priori* assumptions about the evolving response strategies. Our model considers species that both produce a commodity that is beneficial for the other species (following Foster & Wenseleers 2006), a setup that differs from previous pairwise cooperation models (Doebeli & Knowlton 1998; Yamamura *et al.* 2004; Mack 2012) in that local exchange of goods is represented by an interspecific public goods model. After the exchange of goods, individuals may choose to disperse from their local patch conditional upon the cooperativeness received. Such a conditional strategy may result in partner fidelity. Likewise, dispersing individuals can decide whether to settle in a new patch dependent on the cooperativeness of the partner species in that patch, which is equivalent to partner choice. Our aim was to investigate what dispersal patterns results from the joint evolution of conditional dispersal and cooperation and whether these patterns promote the evolution of stable inter-specific cooperation. In our simulations, we focus on a fully symmetric interaction. This allows us to compare the results with those on intraspecific cooperation (Chapter 6) and to study the question whether asymmetric species interactions will evolve from symmetric starting conditions.

THE MODEL

Overview

In a previous model (Chapter 6), we studied the joint evolution of individual cooperativeness and conditional dispersal in a single species. To allow for a comparison of the evolution of within- and between-species cooperation, we here consider a straightforward extension of that earlier model assuming a patch structured environment that is inhabited by species A and B. Generations are discrete and non-overlapping, and both species have a similar life cycle. In a first stage, the interaction phase, individuals play a local cooperation game in which patch members of each species benefit from a commodity produced by the individuals of the other species. Producing this commodity is costly and would hence be selected against in a well-mixed population. The net effect of these costs and the benefits received from the members of the other species determines an individual's condition, which in turn affects its future fecundity in the patch where it will reproduce. In a second stage, the dispersal phase, the individuals of both species may redistribute over the patches. Individuals can make their decisions on whether to leave a patch after evaluating the average 'cooperativeness' of the other species (*i.e.*, the amount of commodities pro-

vided by the patch members of other species). Likewise, they can let their decision on whether to settle in a potential target patch depend on the cooperativeness of the other species there during the interaction phase. Both dispersal and rejection of target patches have mortality costs. In the third and final stage, reproduction, inheritance and population regulation takes place. All individuals settling in a patch (locals and immigrants) produce offspring in proportion to their condition, which reflects the costs and benefits experienced in the interaction phase. After reproduction, the parents die and the offspring of each species compete for a fixed number (N_A and N_B, respectively) of positions in their natal patch. The successful individuals of both species enter a new interaction phase and the remaining individuals die, *i.e.* are removed from both populations.

As in the earlier model, an individual's cooperativeness and its tendencies to disperse and settle elsewhere are heritable strategies that are transmitted from parents to offspring, subject to occasional mutations. Hence, these strategies jointly evolve within each species, and the two species co-evolve over the generations. We considered four scenarios: (1) the dispersal and settlement rates are constant in both species and the same for all individuals (*i.e.*, not heritable); (2) dispersal decisions in both species are based on an evolvable conditional strategy; (3) the settlement rule in both species is based on an evolvable conditional strategy; (4) dispersal and settlement in both species are heritable conditional strategies. The question addressed is whether and under what circumstances conditional dispersal and settlement will favour the evolution of interspecific cooperation.

Interaction phase

Each individual i of species A has a genetically determined 'cooperativeness' x_i, which represents the contribution of i to a commodity X that is beneficial for i's patch members of species B. The total contribution in a given patch, $x_{tot} = \sum x_i$, is distributed over the individuals of species B, yielding an individual share $\bar{x}_B = \frac{1}{N_B} x_{tot}$ per patch member of species B. Likewise, an individual j of species B has a heritable tendency to contribute an amount y_j to a commodity Y beneficial for the other species; each patch member of species A receives a share $\bar{y}_B = \frac{1}{N_B} y_{tot}$ of the total contribution $y_{tot} = \sum y_i$. c_A and c_B represent the respective fecundity costs for the two species per unit of investment, and b_A and b_B denote the respective fecundity benefits per unit of commodity received, so that the fecundities of focal individuals i and j are given by:

$$\begin{aligned} V_i &= V_A + b_A \bar{y}_A - c_A x_i \\ W_j &= W_B + b_B \bar{x}_B - c_B y_j \end{aligned} \qquad (7.1)$$

Here V_A and W_B denote the baseline fecundities of the members of species A and B, respectively. Throughout, we will only consider the case where the two species are fully symmetric with respect to their fitness parameters: $c_A = c_B = c$, $b_A = b_B = b$, $V_A = W_B$, and $N_A = N_B = N$. In our model, only relative fecundities (in comparison to other patch members of the same species) are important. We can therefore normalize fecundities by dividing all terms in equation (7.1) by b:

$$\begin{aligned} v_i &= v_A + \bar{y}_A - (c/b)x_i \\ w_j &= w_B + \bar{x}_B - (c/b)y_j \end{aligned} , \tag{7.2}$$

where $v = V/b$ and $w = W/b$. In our simulations, we will systematically vary the cost-benefit ratio c/b while keeping the normalized baseline fecundities fixed at $v_A = w_B = 5$.

Dispersal and settlement

In the dispersal phase, each individual has the opportunity to leave its patch of origin. Depending on the scenario considered, the dispersal probability d is either constant ($d = 0.15$; the value predicted by Taylor (1988)) or an evolvable conditional strategy where an individual's dispersal probability is made dependent on the cooperativeness of the patch members of the other species. As in Chapter 6, we consider conditional strategies that are given by a logistic function. Individuals of species A make their probability to leave the natal patch dependent on $\bar{y}_A$, *i.e.* on their share in the commodity Y produced by the patch members of species B:

$$d(\bar{y}_A) = \frac{1}{1 + \exp(-\beta_d \cdot (\bar{y}_A - \alpha_d))} . \tag{7.3}$$

Here α_d and β_d are heritable parameters that characterize the individual's 'norm of reaction' (see Figure 6.1). If an individual harbours a positive β_d ('type 1' strategy), it has the tendency to leave productive patches (large values of $\bar{y}_A$), while individuals with a negative β_d ('type 2') tend to leave patches with a low productivity. When β_d is large in absolute value, the dispersal strategy approaches an all-or-nothing strategy, where an individual will always leave or always stay, depending on whether $\bar{y}_A$ is larger or smaller than α_d. Similarly, the dispersal tendency of members of species B is determined by evolvable parameters α_d and β_d, but now the dispersal decision depends on $\bar{x}_B$ and thus on the cooperativeness of the patch members of species A.

Dispersing individuals encounter patches at random. Depending on the settlement scenario considered they either settle on the first patch encountered or they base their settlement decision on an evolvable conditional evaluation of candidate patches. In the latter case, the probability to settle in a candidate target patch depends on the cooperativeness of the other species in that patch. For an individual of species A, the settlement probability is given by:

$$s(\bar{y}_A) = \frac{1}{1 + \exp(-\beta_s \cdot (\bar{y}_A - \alpha_s))} \tag{7.4}$$

where again α_s and β_s are heritable parameters. Similarly, individuals of species B make their settlement decisions depending on $\bar{x}_B$.

Dispersal and the rejection of patches are costly: the viability of dispersing individuals is reduced by a factor $1 - m_d$, while each time that a possible target patch is rejected the viability is further reduced by a factor $1 - m_s$. Dispersal-related mortality m_d and rejection-related mortality m_s were assumed to be the same for both species and given $m_d = 0.1$ and $m_s = 0.001$.

Reproduction and inheritance

After all individuals have settled, the members of each patch reproduce according to the fecundity accrued in their natal patch. Of the offspring produced in each patch, N_A and N_B are chosen at random to form the patch members that will enter the next social interaction phase, whereas all parents and all other offspring die.

Individuals are haploid and reproduce clonally, so that, each individual is characterized by alleles at five jointly evolving loci. Allelic values are real numbers that determine the individual's cooperativeness and its condition dependent dispersal and settlement strategy, *i.e.* the parameters [(α_d, β_d) and/or (α_s, β_s)]. When reproducing, parents transmit all five alleles to their offspring. At each locus, mutations occur with probability $\mu = 0.01$. In such cases a small number drawn from the uniform distribution on the interval (–0.1, +0.1) is added to the allele at the corresponding locus. See Chapter 6 for further details.

Simulation settings

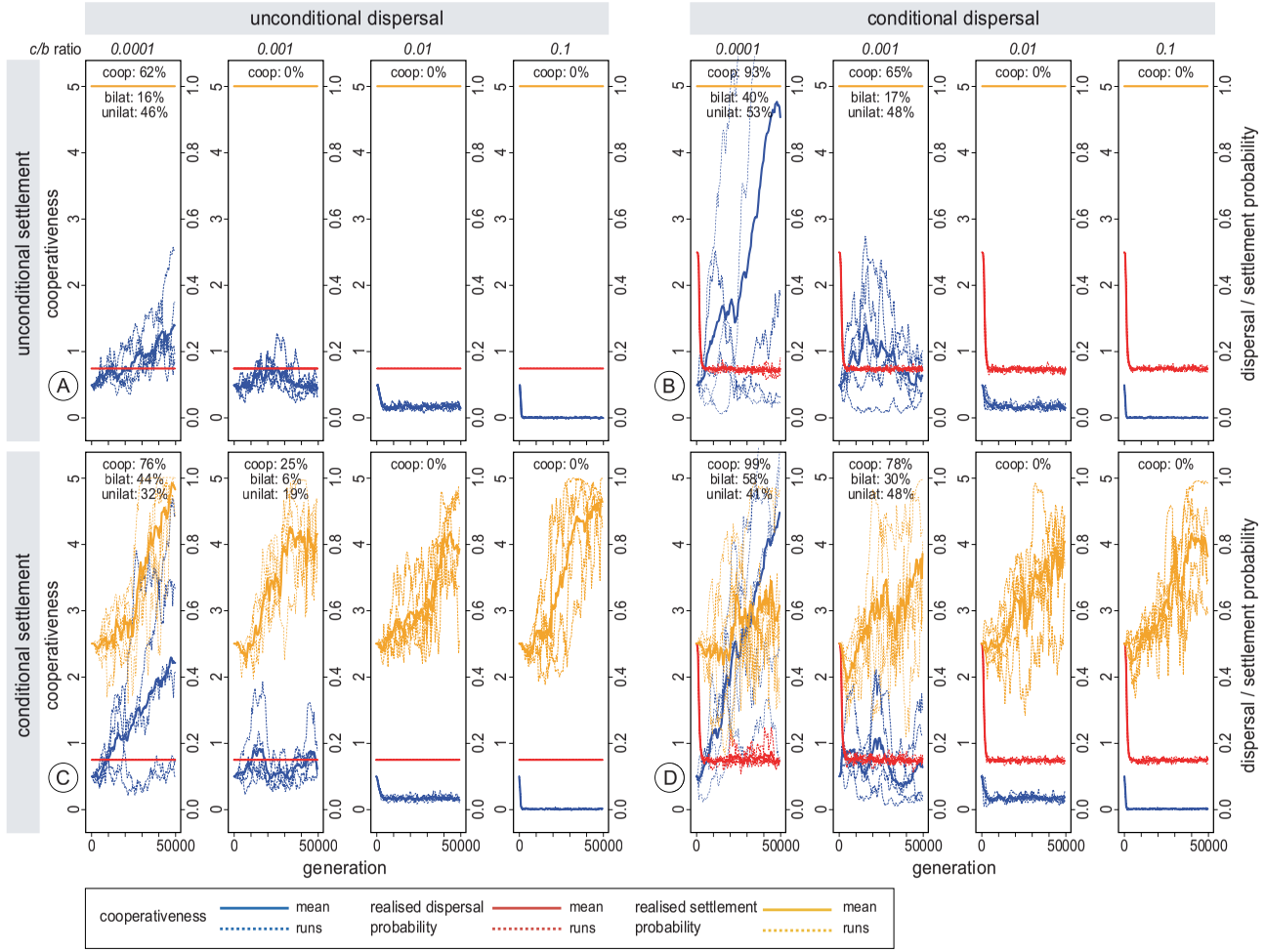
We simulated populations consisting of 25,000 individuals per species that where distributed over 1,000 patches, with each patch harbouring $NA = N_B = 25$ individuals per species. All individuals were initialized at the allelic values $x = \alpha_d = \alpha_s = 0.5$ and $\beta_d = \beta_s = 0$. We considered four different cost-benefit ratios ($c/b = 0.0001, 0.001, 0.01, 0.1$) for all four dispersal and settlement scenarios and we ran 100 replicate simulations for each scenario and parameter combination. Most simulations were run for 50,000 generations, but we regularly ran longer simulations (>200,000 generations) to check the robustness of our results.

The simulation program was coded in C^{++} (code available upon request from A.I.), and compiled in g^{++} (4.3.4; Free Software Foundation, Inc. 2008).

RESULTS

Does conditional dispersal promote interspecific cooperation?

Figure 7.1 summarizes the results of our simulations on the evolution of between-species cooperation. Our two-species model strongly resembles the public goods model for within-species cooperation considered earlier (Chapter 6). In that earlier model we concluded that conditional dispersal can hamper (within-species) cooperation for low cost-benefit ratios, while it strongly promotes cooperation when cost-benefit ratios are high. In contrast, Figure 7.1 reveals that – irrespective of the dispersal scenario – between-species cooperation never got off the ground for the two highest cost-benefit ratios considered ($c/b = 0.01, 0.1$). When the cost-benefit ratio was extremely low ($c/b = 0.0001$), cooperativeness slowly increased even when neither dispersal nor settlement decisions were evolving conditional strategies (Figure 7.1A): in 62% of the 100 replicate simulations at least one of the two species reached a cooperativeness level of 2 within 50,000 generations. We chose this (arbitrary) threshold to classify whether or not 'cooperation' had evolved in a species. Both conditional dispersal and conditional settlement had a significant effect on the evolution of cooperation. When conditional dispersal could evolve (Figure 7.1B) the percentage of simulations leading to cooperation in at least one species increased from 62% to 93% ($P < 0.001$, Fisher exact


unconditional dispersal
conditional dispersal
c/b ratio
0.0001
0.001
0.01
0.1
unconditional settlement
conditional settlement
coop: 62%
bilat: 16%
unilat: 46%
coop: 0%
coop: 93%
bilat: 40%
unilat: 53%
coop: 65%
bilat: 17%
unilat: 48%
coop: 76%
bilat: 44%
unilat: 32%
coop: 25%
bilat: 6%
unilat: 19%
coop: 99%
bilat: 58%
unilat: 41%
coop: 78%
bilat: 30%
unilat: 48%
A
B
C
D
cooperativeness
dispersal / settlement probability
generation
0
50000
cooperativeness
mean
runs
realised dispersal probability
realised settlement probability

test), while the percentage increased to 76% when conditional settlement could evolve (Figure 7.1C; $P < 0.016$, Fisher exact test). Conditional dispersal and conditional settlement had a synergistic effect in that the percentage of simulations leading to cooperation when both were conditional increased to 99% (Figure 7.1D; $P < 0.015$ for the comparison of 93% and 99%, Fisher exact test). In contrast, the evolution of conditional settlement had no marked effect on the evolution of within-species cooperativeness (Chapter 6).

For the intermediate cost-benefit ratio $c/b = 0.001$ (which is still very low) the simulation results were somewhat mixed. In the absence of conditional dispersal and conditional settlement (Figure 7.1A), the cooperativeness of both species stayed well above zero in all simulations, but no species reached the threshold criterion of 2. The percentage of cooperation increased to 25% in case of conditional settlement (Figure 7.1C; $P < 0.001$, binomial test), to 65% in case of conditional dispersal (Figure 7.1B; $P < 0.001$, binomial test), and to 78% in case of conditional dispersal and conditional settlement (Figure 7.1D; $P < 0.021$, Fisher exact test). However, in none of the simulations did cooperativeness reach high levels, and the occasional (small) outbursts in cooperativeness were in all cases followed by a subsequent drop in cooperativeness to small values.

Since consistent and sizeable cooperation levels only evolved when the cost-benefit ratio was extremely low ($c/b = 0.0001$), we will focus on this case for the rest of this paper. In view of equation (7.2), between-species cooperation is very 'cheap' in this case, because even if an individual contributes 10 units to the commodity that is beneficial to the other species (a value that was almost never reached in our simulations), the loss in fecundity (0.001) is minute in comparison to the baseline fecundity ($v_A = w_B = 5$). One might therefore suspect that evolution is largely governed by random factors (genetic drift and mutation), but this does not seem to be the case. When simulations were run for a longer period (Figure 7.2 shows two representative examples), the evolution of cooperativeness showed a clear trend that was only marginally affected by stochastic fluctuations. As we will show below, stochasticity was mainly important for determining which kind of strategies evolved in the two species in a given simulation run.

Evolution of asymmetric species interactions

To make our results comparable with the situation of within-species cooperation considered earlier, we consider two species that are identical in all respects, including fitness

Figure 7.1 (left) Effect of the cost-benefit ratio on the evolution of interspecific cooperation under different dispersal and settlement scenarios. Evolution of cooperativeness (blue), mean dispersal probability (red) and mean settlement probability (orange) for four cost-benefit ratios and four dispersal scenarios: (A) fixed dispersal probability ($d = 0.15$) and fixed settlement probability ($s = 1.0$); (B) evolvable conditional dispersal strategy and fixed settlement probability; (C) fixed dispersal probability and evolvable conditional settlement strategy; (D) evolvable conditional dispersal and settlement strategies. Each plot shows 5 randomly selected replicate simulation runs (dashed lines) and their means (bold lines). Left y-axis: cooperativeness; right y-axis: probability of dispersal and settlement in a new patch. The graphs in the top of each panel refer to the percentage of 100 simulation runs during 50,000 generations where the cooperativeness of one or both species exceeded the (arbitrary) threshold value of 2, meaning that we obtained: 'cooperation', when at least one species exceeded the threshold; 'bilateral cooperation', when both species exceed the threshold; and 'unilateral cooperation', when only one species exceeded the threshold.

parameters, population sizes and initial conditions. Yet, as exemplified by Figure 7.2, the level of cooperation that evolved in the two species widely diverged. This is confirmed by Figure 7.1, which indicates that bilateral cooperation (where both species reached cooperativeness levels above 2) emerged only in a fraction of the 'cooperative' simulation runs. In case of unconditional settlement (Fig 7.1AB), the majority of cooperative simulation runs were classified as 'unilateral', that is, they corresponded to a simulation where only one of the two species exceeded the threshold cooperativeness value of 2 within 50,000 generations. Interestingly, conditional settlement had a positive effect on bilateral cooperation, as the percentage of bilateral cooperation increased significantly, irrespective of whether dispersal happened at a fixed rate (44% versus 16%; $P < 0.001$, Fisher exact test) or was an evolving conditional strategy (58% versus 40%; $P < 0.005$, Fisher exact test).

For dispersal scenario 4 (the evolution of both conditional dispersal and conditional settlement), Figure 7.3 depicts the cooperativeness evolved in both species for ten randomly chosen simulation runs. While it is apparent that in each simulation a sizeable level of cooperation evolved in at least one species, the other species typically lagged far behind and sometimes did not evolve cooperativeness at all. Only in one of the simulation runs

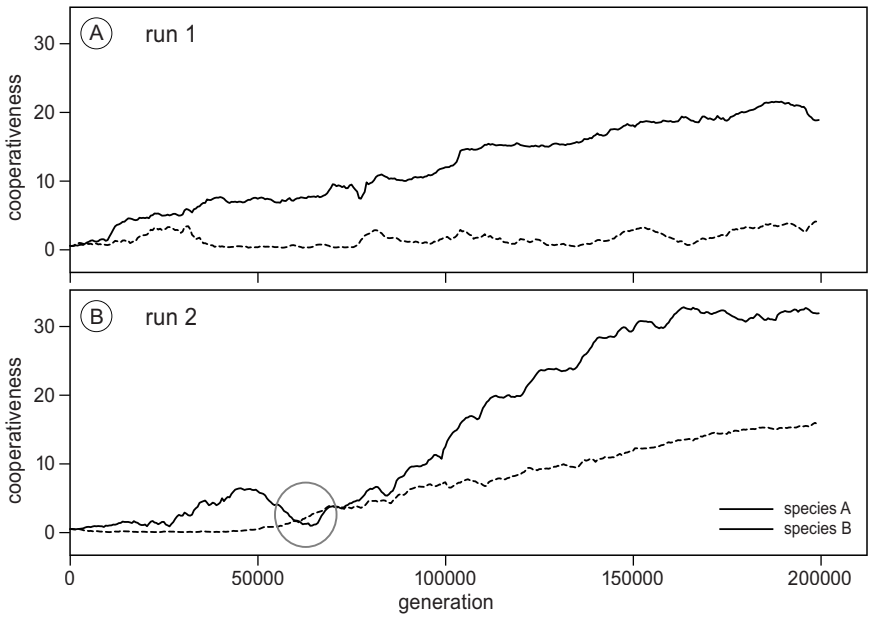


Figure 7.2 Evolution of asymmetric cooperation. Long-term evolution (200,000 generations) of cooperation in species A (solid line) and species B (dashed line). In both example runs, species A happens to evolve a high degree of cooperativeness. Cooperation remains unilateral in (A) where even after extended periods of time cooperativeness does not get off the ground in species B. Cooperation remains asymmetric but becomes bilateral in (B), where after a long period without cooperation, cooperativeness starts to increase steadily in species B. After almost 10,000 generations (indicated by the circle), the relationship between A and B reversed for a brief period of time. In both simulations: $c/b = 0.0001$ and we allow for dispersal scenario 4 with both conditional dispersal and conditional settlement.

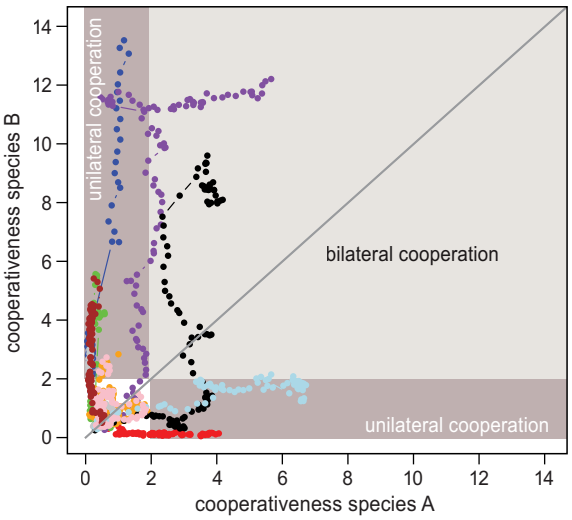


Figure 7.3 Evolutionary trajectories resulting in unilateral and bilateral cooperation. The cooperativeness of both species is shown for ten randomly chosen example runs for the cost-benefit ratio $c/b = 0.0001$ and dispersal scenario 4 with a simulation time of 50,000 generations. Based on our classification of a species as 'cooperative' when its level of cooperativeness exceeded a threshold value of 2, the graph is subdivided into four sections: (1) bottom-left corner (white): no species is cooperative; (2) bottom-right section (brown): unilateral cooperation by species A; (3) top-left section (brown): unilateral cooperation by species B; (4) top-right section (grey): bilateral cooperation.

(black dots), did the cooperativeness of both species consistently stay above our classification threshold of 2. In one other simulation (purple), a long period of asymmetry ended in a more symmetric outcome in generation 50,000. We conclude therefore that the evolution of symmetric bilateral cooperation was the exception rather than the rule. A more typical outcome was unilateral cooperation, where one species reaps the benefits of cooperation while not paying the (even very low) costs of being cooperative itself.

Do partner choice and partner fidelity promote the evolution of cooperation?

The heat plots in Figure 7.4, based on two of the simulations of Figure 7.3, illustrate how the distribution of alleles for cooperativeness (left panels in Figure 7.4A and Figure 7.4B) and the parameters β_d (middle panels) and α_d (right panels) that characterize the conditional dispersal strategy evolve in the two species (Figure 7.4C and Figure 7.4D). Figure 7.4A (= black dots in Figure 7.3) shows a simulation run where relatively high values of cooperativeness evolved in both species. Since the dispersal strategy is conditional on the cooperativeness of the *other* species, the top panels show β_d and α_d of species B next to the cooperativeness of species A, while the bottom panels illustrate the dispersal parameters of species A next to the cooperativeness of species B. The dispersal parameters of species A show a consistent pattern: the switch point α_d of the dispersal function (equation 7.3)) evolves in parallel with the cooperativeness y of species B and assumes more or less the same values. From the start, β_d takes on positive values, implying that a type-1 dispersal function gets established in species A (shown for generation $t = 10000$ in red in

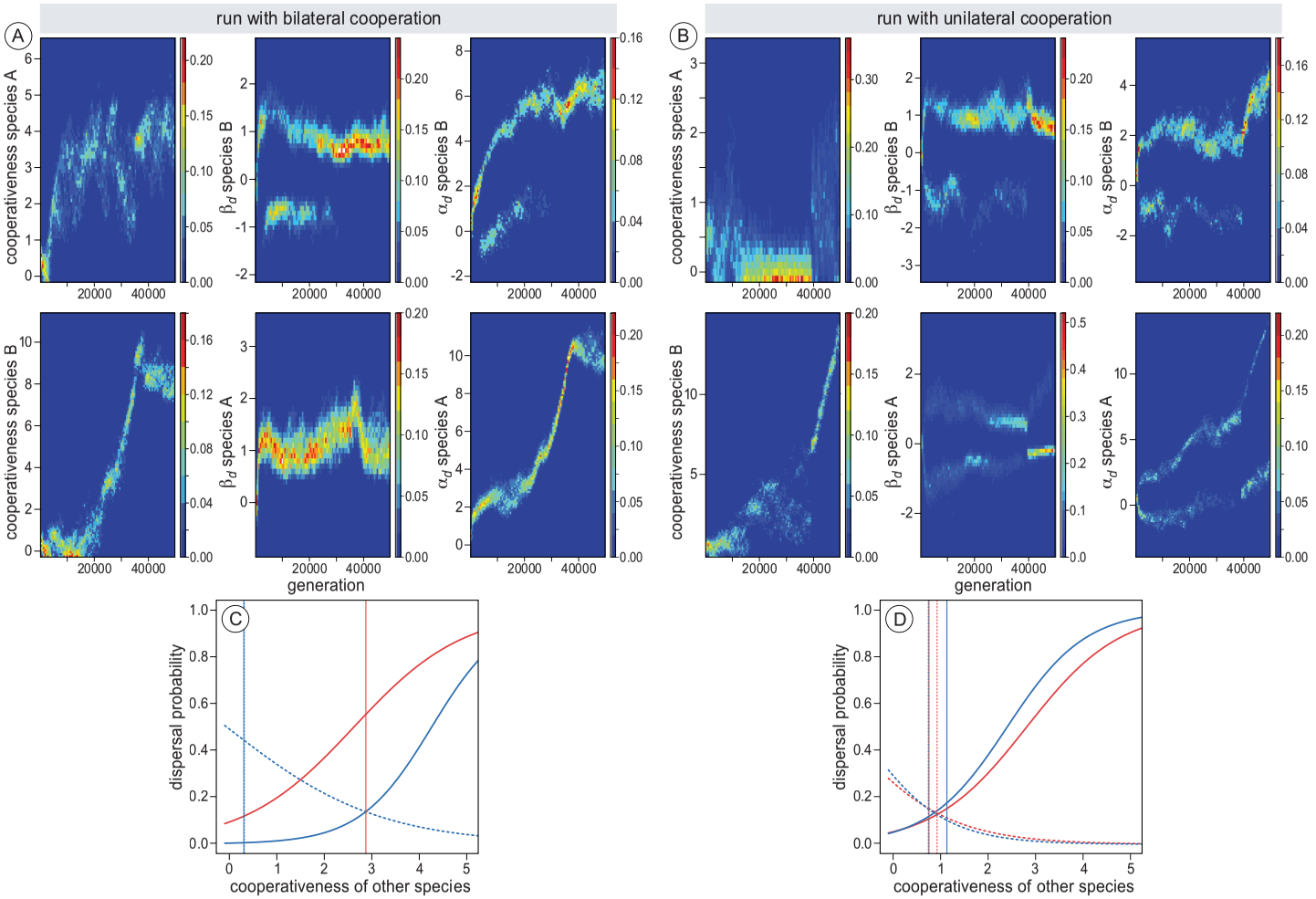

run with bilateral cooperation
run with unilateral cooperation
A
B
C
D
cooperativeness species A
cooperativeness species B
β_d species B
α_d species B
β_d species A
α_d species A
generation
dispersal probability
cooperativeness of other species

Figure 7.4C). This means that the individuals in species A tend to leave patches with a high degree of cooperativeness of the other species, while they tend to stay when cooperativeness is low. In the longer run, species B follows the same pattern, but two different dispersal strategies (a type-1 and a type-2 strategy) coexist in the first 25,000 generations (Figure 7.4C, blue lines) until the type-1 strategy finally outcompetes the other. Both species evolved a type-1 settlement strategy (not shown), implying that individuals tend to accept patches with an above-average degree of cooperativeness of the other species, while they reject non-cooperative patches.

The simulation in Figure 7.4AC exemplifies a few general points. Whenever a species evolved a high degree of cooperativeness, the jointly evolved dispersal strategy and the jointly evolved settlement strategy were both type-1. In case of the settlement strategy, this is perhaps not surprising, since it corresponds to the expected outcome of partner choice, *i.e.* accept cooperative neighbourhoods and reject non-cooperative environments. However, the evolved dispersal pattern is not in line with the standard expectation concerning partner fidelity, although the observed pattern appears to be sensible. An individual that stays in a patch with a high cooperativeness of the other species has a fecundity benefit, but it also has to compete with conspecifics that receive a high benefit as well. It is therefore advantageous to leave and to 'cash' the benefits of cooperation in a less competitive patch, because all patches will ultimately be downregulated to two times 25 individuals in the next generation.

Figure 7.4B (= blue dots in Figure 7.3) exemplifies the joint evolution of cooperativeness and cooperation-dependent dispersal in a simulation run with a strong evolved asymmetry between the two species. In line with the above considerations, species B, which evolves a high level of cooperation, makes use of a type-1 dispersal strategy. However, a second dispersal strategy of type-2 managed to coexist for extensive periods of time (for generation $t = 10000$, Figure7.4D, dashed blue line). In species A, cooperativeness did not really get off the ground, but there was considerable variation in low-level cooperativeness (for generation $t = 10000$, Figure 7.4D, red vertical lines). The (relatively) cooperative individuals of species A harboured a type-1 dispersal strategy, while a low-level of cooperation was associated with a type-2 dispersal strategy. Both species evolved a type-1 settlement strategy (not shown). This example demonstrates that cooperation can be both strongly asymmetric between species and have considerable variation in cooperativeness within species and that the response to the other species' cooperativeness can persist for extensive periods of time.

Figure 7.4 (left) Joint evolution of cooperativeness and conditional dispersal in replicate simulation runs leading to either the evolution of bilateral (A, C) or unilateral cooperation (B, D). The panels are heat plots illustrating for both species the evolution of the frequency distribution of the alleles for cooperativeness and the parameters β_d and α_d characterizing the conditional dispersal strategy. In the upper parts of the figure (A, B), the top panels show the cooperativeness of species A (left) and the dispersal parameters β_d (middle) and α_d (right) of species B. The bottom panels show the cooperativeness of species B (left) and the dispersal parameters of species A (middle and right). For generation 10,000 panels (C) and (D) show representative dispersal strategies (red, species A, blue, species B; solid lines: Type-1 strategy, dashed lines, Type-2 strategy) and the average cooperativeness of the individuals expressing these strategies (vertical lines).

DISCUSSION

Cost-benefit effects on the evolution of interspecific cooperation

In our model cooperation between species only evolved when the cost-benefit ratio of the interaction was extremely low. Under higher cost-benefit ratios, no cooperation emerged, irrespective of the dispersal scenarios considered (Figure 7.1). This outcome contrasts the results obtained in Chapter 6, where we showed that conditional dispersal promotes within-species cooperation particularly strongly under high cost-benefit ratios. This is because, in both models, the only adaptive response as a cooperative individual under high cost-benefit ratios is to evolve competitor avoidance. However, under intraspecific cooperation (Chapter 6), the cooperative individual in fact just helped the competitors becoming competitive herself. In case of interspecific cooperation the competitors to be avoided are not the same individuals that she also cooperates with. Cooperation between species will therefore not come off the ground when both species have a strong tendency (as selected for by high c/b ratios) to avoid conspecific competitors and leave beneficial environments created by the other species.

With the extremely low cost-benefit ratios required for mutualism in our current model, the mutualistic relationships that evolved may be viewed as examples of by-product mutualism, where partners exchange goods that are cheap (or even free) to produce but yield high benefits for the other species (Connor 1995; Leimar & Connor 2003; Leigh 2010). A well-known example of by-product mutualism is honeydew provided by aphids to ants, which is based on trading waste product for protection and obtaining the extra bonus of engaging waste collectors that enhance hygiene in the aphid colony (Stadler & Dixon 2008). Also others have found that (low) c/b ratios enhance the probability (Yamamura *et al.* 2004; Foster & Wenseleers 2006), but it would be useful for future studies to let cost-benefit ratios jointly evolve with cooperativeness and conditional dispersal, as it is conceivable that costs and benefits change over evolutionary time as mutualistic partners co-evolve. Evolving cost-benefit ratios did indeed promote mutualism in a model of pairwise interspecific cooperation (Doebeli & Knowlton 1998).

Partner fidelity, partner choice and the evolution of mutualism

Provided cost-benefit ratios were sufficiently low, interspecific cooperation evolved in all four simulated dispersal scenarios. However, cooperation in at least one of the species evolved more frequently when conditional dispersal (equivalent to partner fidelity) was allowed to evolve as well (Figure 7.1AB). Also the joint evolution of conditional settlement (equivalent to partner choice) promoted cooperation (Figure 7.1AC) and we observed an additional synergistic effect of conditional dispersal and settlement that increased the frequency in which bilateral cooperation evolved (Figure 7.1D; see below). Previously, partner fidelity and partner choice have theoretically been shown to promote mutualism (Foster & Kokko 2006; Foster & Wenseleers 2006), but, these models assumed that individuals would have an innate tendency to leave non-cooperators and move towards cooperators. Such positive conditional settlement could indeed evolve in our model, but conditionally dispersing individuals evolved to leave cooperative patches rather than non-cooperative ones. These counter-intuitive results match findings in our similar model on

within-species cooperation (Chapter 6). Although unexpected at first these within-species results could be explained by competition avoidance: by leaving 'good' patches, individuals avoided competition over reproduction with individuals they themselves helped to be strong competitors. Virtually the same explanation applies to the current model for inter-specific cooperation. A tendency to stay in 'good' patches is clearly not advantageous in our model; individuals would profit from the cooperativeness of the other species by having a higher fecundity, but their offspring would face fierce competition for the fixed number of positions available since all conspecific patch members would be highly fecund as well. In other words, the benefits received in a good patch can only be 'cashed' elsewhere. This effect may have been buffered for when we would have allowed for local resource enhancement to take effect: if patch sizes would increase with patch productivity rather than being constant.

Emergence of variation in cooperativeness and conditional strategies

To allow comparison of our results on between-species cooperation with those obtained for within-species cooperation in Chapter 6, we focused on a symmetric situation in which both species were virtually identical and started simulations with the same initial conditions. Still, a remarkable degree of interaction asymmetry evolved, with the majority of the runs resulting in unilateral rather than bilateral cooperation (Figure 7.3). Doebeli and Knowlton (1998) showed as well that symmetric starting conditions can yield fluctuating end results for the evolution of mutualism. Most likely, similar or even higher degrees of asymmetry would evolve when the model contains asymmetry *a priori*. However, given some of the other counterintuitive results, it would seem wise to explicitly test this expectation. Such future studies would be particularly relevant because many mutualisms are characterized by asymmetry between partners, for example in body size, generation time, and number of interacting individuals (reviewed in Leigh 2010).

Throughout our simulations we maintained considerable variation for cooperativeness both between and within species and the same was true for conditional strategies. Over extended periods of time, opposite dispersal strategies can apparently coexist (Figure 7.4), often in combination with differing levels of cooperativeness. The importance of variation for beneficial effects of conditional strategies has previously been recognized in different contexts (Ferrière *et al.* 2002; Foster & Kokko 2006; McNamara *et al.* 2008; Wolf *et al.* 2011). Indeed, costly conditional behaviour requires variation; it is only worth paying the costs of being choosy or responsive if these costs are balanced by fitness benefits emanating from making good choices.

Acknowledgements

We thank Yiming Yu, Joke Bakker and Marten van de Sanden for help with the development and implementation of the model. Support for this work was obtained through a PhD felolowship from the Centre for Ecological and Evolutionary Studies, University of Groningen and by grants from the Langerhuizen Fund and Nicolaas Mulerius Fund, awarded to ABFI. The Centre for High-Performance Computing and Visualisation at the University of Groningen provided access to the Millipede High Performance Cluster.

CHAPTER 8

Epilogue: what have we learned about farming mutualism?

Aniek B.F. Ivens

In this thesis, I have studied the problem of the evolution of stable (farming) mutualism by means of combined empirical (Chapters 2-5, Box A, B) and theoretical approaches (Chapters 6, 7). For a detailed description of my findings I refer to the individual chapters. Instead, in this last chapter I will reflect on what we have learned about farming mutualisms in general and the suitability of the *Lasius flavus*-root aphid system as model system for farming mutualisms in particular. I will also look ahead to future avenues in both empirical and theoretical studies on farming mutualism by discussing potential future empirical angles and by describing the characteristics of a theoretical model specifically tailored to farming mutualism.

ARE CONDITIONAL BEHAVIOUR AND LOW COSTS THE KEYS TO SUCCESS IN COOPERATION?

How cooperation can evolve by means of natural selection is not self-evident. In a world in which individuals are selected to outcompete others, even a small cost of cooperative behaviour can be too high and prevent its evolution (see also Chapter 1). Previously it was shown that selectively continueing cooperative interactions (*partner fidelity*) or selectively engaging in cooperative interactions (*partner choice*) can be important mechanisms to promote the evolution of cooperation (among others Trivers 1971; Bull & Rice 1991). Evolution of such conditional mechanisms is often hard to predict. Indeed, when modelling conditional behaviour one easily obtains counter-intuitive results (*e.g.* Van Doorn *et al.* 2003; Pen & Taylor 2005; Fawcett *et al.* 2011). This was also the case in Chapter 6, in which conditional dispersal (partner fidelity in our model) was shown to promote within-species cooperation, but did so in unexpected ways: (1) cooperation evolved when individuals tended to leave cooperative patches for non-cooperative ones, rather than the other way around, (2) conditional dispersal could also lead to the disappearance of cooperation and (3) the evolution of cooperation under conditional dispersal evolved more easily when cost-benefit ratios were high. As explained in Chapter 6, although counter-intuitive at first, these results can well be understood by taking local resource enhancement into consideration. I therefore conclude that when investigating the importance of conditional behaviour for the evolution of cooperation one should be careful to not only focus on the cost-benefits of the cooperation pay-off, but also to take into account other effects such as those generated by population regulation.

Cooperation between species, however, is expected to be even more difficult to evolve than the within-species cooperation studied in Chapter 6. This is especially so because no kin-selective advantage can take effect in an interaction between individuals of different species (see Chapter 1). Indeed, in my model in Chapter 7, between-species cooperation only evolved under much more restricted conditions than within-species cooperation in the comparable model of Chapter 6. In particular, interspecific cooperation could only evolve under very low cost-benefit ratios, in which there are hardly any costs involved in engaging in an interaction (comparable to by-product mutualism). Given these low costs, the model in Chapter 7 showed that conditional dispersal (partner fidelity) can also promote the evolution of between-species cooperation, in particular in combination with additional condi-

tionality in the form of conditional settlement (partner choice).

Interspecific cooperation thus evolves most easily under very low cost-benefit ratios and under conditions in which partner fidelity and partner choice can interact. The conceptual models of Chapters 6 and 7, however, did not consider specific cooperative systems, rendering the underlying assumptions rather general and biologically less realistic. Future extensions of these conceptual models should include a wider range of cooperative games, such as one-to-one interactions and interactions with asymmetry in cost-benefit ratios and information available to participants, in order to test whether these general results are robust. Moreover, for a better theoretical understanding of the evolution of farming mutualism, we will need to go beyond conceptual models and develop more mechanistic models specifically tailored to farming mutualism. In the section on 'farming mutualism', I will also give a detailed prospect of the contours of such a model. First, I will discuss what we have learned about the specific farming mutualism between Yellow meadow ants and their root aphids and discuss its prospect as a future model system for farming mutualism.

EVALUATION OF *L. FLAVUS*- SYSTEM AS A STUDY SYSTEM FOR FARMING MUTUALISMS

At the start of my dissertation project, very little was known about the *L. flavus* – root aphid system. All information was based on a handful of studies, published between the 50's and early 90's (Pontin 1958; 1959; 1961b; Paul 1977; Godske 1991; 1992; Smart 1991). In addition, some information seemed to be based on anecdotal evidence and none of the studies or observations had been performed in my main study population on the island of Schiermonnikoog, the Netherlands. Nevertheless, for several reasons the *L. flavus* system seemed a promising study system for farming mutualism: (1) field and laboratory studies seemed quite feasible; a large study population was available and laboratory setups had been described in the literatur (Muir 1960; Dixon 1970; Paul 1977; Smart 1991); (2) the available information suggested considerable life-history variation among the symbiont root aphid species that would enable comparative studies; and (3) a detailed study of conflict resolution mechanisms in farming mutualism seemed feasible, because of the available variation in symbiont life-history with respect to for example myrmecophily (dependence on ants) and reproductive mode. In this section, I will evaluate these three notions in light of what we know now and discuss the future potential of this ant-aphid interaction as a model system for farming mutualism.

The feasibility of field and laboratory studies

During the first year of this project, I visited a large number of field sites in Northwest Europe (the Netherlands, Belgium, Germany and Denmark, see Figure 8.1, Table 8.1). These site visits were aimed at obtaining insight into aphid species diversity in *L. flavus* nests in general and at evaluating the suitability of these sites for my studies. The salt marsh population on the island of Schiermonnikoog proved to be the most suitable location with large numbers of ant mounds, large numbers of aphids per mound, and a considerable

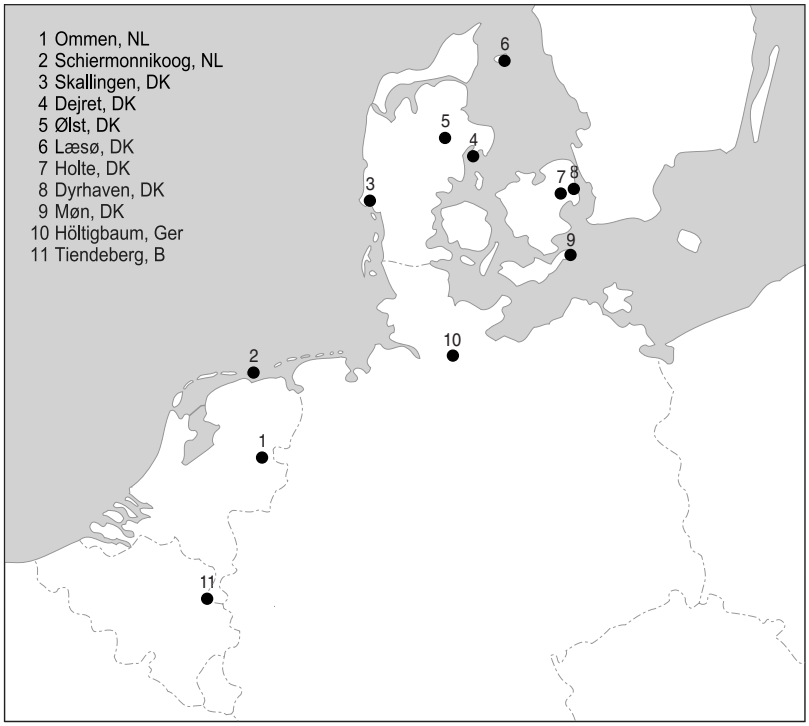


Figure 8.1 Overview of sample locations in the Netherlands, Denmark, Germany and Belgium. Map courtesy of D. Visser.

diversity of aphid species. The field survey further showed that the aphid diversity on Schiermonnikoog was comparable to the diversity on the Danish island of Læsø, but that inland and salt marsh communities differed in aphid species composition (see Table 8.1).

The large ant population on Schiermonnikoog allowed for systematic sampling of root aphids from ant mounds. For aphid sampling I mainly followed Pontin (1978) and Godske (1991; 1992), using a fixed scheme for taking soil samples (Figure 4.1). However, in contrast to the previous studies which used Tullgren funnel sorting for separating aphids from the soil samples, I hand-sorted aphids from the soil. This method allowed me to separate aphids according to aphid chambers, which for the first time opened the possibility of analysing aphid (genetic) diversity at the chamber level. As documented in Chapter 4, it turned out that aphid chambers hardly ever contain multiple species and multiple clones per species. The downside of hand-sorting is that I possibly missed a number of aphids which might have been detected by Tullgren sorting, especially early developmental stages (of all species) that were too small to detect by eye (for a discussion, see the appendix of Chapter 4).

Lasius niger, a sister species of *L. flavus*, and its above-ground facultative aphid symbionts (in particular the pea aphid *Aphis fabae*) has been studied in controlled greenhouse experiments (Offenberg 2001; Vantaux *et al.* 2011a; 2011b; 2012). This greenhouse set-up

allows for the study of fundamental characteristics of the mutualistic relationship, such as partner choice (Vantaux *et al.* 2012), the effects of aphid clone and ant attendance on honeydew quality (Fischer & Shingleton 2001; Yao & Akimoto 2001; Vantaux *et al.* 2011b), ant suppression of aphid dispersal (Oliver *et al.* 2007), and shifts from aphid milking to preying on aphids (Offenberg 2001). However, these results are restricted to a facultative, above-ground interaction and a full understanding of the dynamics of ant farming mutualism requires comparison with the results obtained in an obligate, underground system. A laboratory set-up of the *L. flavus* system would allow such a comparison. Unfortunately, culturing ants and aphids in the laboratory turned out to be very challenging. Earlier records by Muir (1960), Dixon (1970), Paul (1977) and Smart (1991) showed that culturing aphids and ants in a combined set-up can be done, but that aphid numbers often remain very small. Our pilot efforts showed that *L. flavus* colonies (with queens, transferred from the field) can best be kept in separate boxes on a diet of meal worms and honey solution, with medium humidity and daily fluctuating temperature, to mimic natural conditions (A. B. F. Ivens and C. F. Veen, personal observation). Aphids were best kept in tubes with a small number of ant workers on roots of *Festuca rubra* (Figure 8.2).

When aphids were kept without ant workers, they quickly died because their trophobiotic organ got infected with mold when not being cleaned from honeydew by ants. In addition, ant workers air the soil by constant digging, which might also be important for root

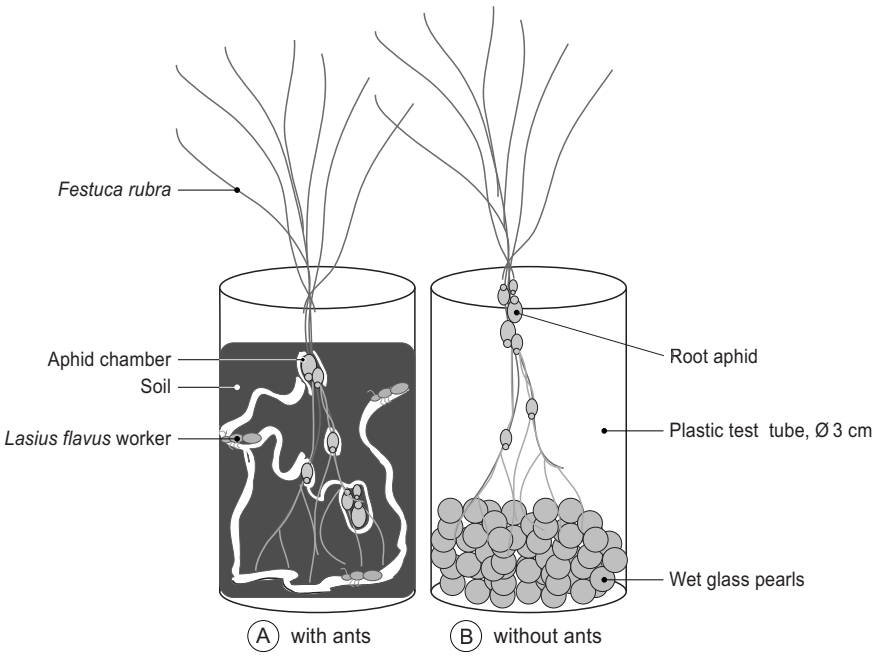


Figure 8.2 Set-ups for laboratory culturing of root aphids with ant workers (A) and without ant workers (B). In both set-ups, grass *Festuca rubra* is grown in plastic experimental test tubes and aphids feed on the grass roots. Note that in set-up (A) with the grass rooted in soil, aphids do not survive without ant workers airing the soil and cleaning their abdomen. In set-up (B) aphids survived approximately two weeks.

aphid survival. In our pilot studies, neither ants nor aphids reached densities high enough for larger-scale experiments. Future efforts to culture ants and aphids should include culturing aphid on *Holcus lanatus* (Paul 1977) and direct connection of root aphids and ant colonies, which has previously been shown to yield higher aphid numbers for experimental and taxonomic studies. Alternatively, experiments can be performed with ants and aphids directly after being collected from the field. This method was applied in the pilot experiments of Box A of this thesis, in which I studied aphid and mealybug acceptance by ant sub-colonies. Although possible, this collection method does not allow for carefully controlled laboratory circumstances and sample sizes.

Diversity in the *L. flavus* system

Over the course of my PhD studies, I found 15 different species of root aphids in *Lasius flavus* nests and five species of underground mealybugs (Table 8.1). These aphid species have all been described before from *L. flavus* nests in Britain and Denmark (Paul 1977; Pontin 1978; Heie 1980; Godske 1991), but as far as I am aware, this is the first detailed study of root aphids and mealybugs in *L. flavus* nests in the Netherlands. For species identification I used a protocol for microscopic preparation (modified from Heie 1980). This process is very time consuming and identification remains uncertain, because of the limited number of records on root aphid taxonomy available (Zwölfer 1958; Paul 1977; Heie 1980). It is therefore likely that some cryptic species remained undetected; this issue can only be resolved by further genetic analysis, following up on our initial work presented in Chapter 5.

The sparse records on the species found indicated a large variation in life-history characteristics. For example, *Anoecia corni* and *Tetraneura ulmi* were assumed to complete their sexual cycle in Northwest Europe on their primary host plants *Cornus* and *Ulmus* resp., whereas *Forda* spp., *Geoica* spp. and *Anoecia zirnitsi* have been inferred to reproduce only anholocyclically in the area (Paul 1977; Heie 1980). Also the extent to which these root aphids disperse differs between species according to the few available records: some species obligately leave ant nests in search for their primary host plants to reproduce, for others clonal alates (winged individuals) have been described and others are assumed to never leave the ant nest (Paul 1977; Heie 1980). Lastly, these aphid species are assumed to differ significantly in their dependence on ants: *Forda* spp., for example, have been described to be obligate myrmecophiles whereas *Tetraneura ulmi* is considered a facultative myrmecophile by some (Paul 1977; Heie 1980). In Chapters 2 & 3, I first tried to verify early records on root aphid reproduction and dispersal using microsatellite markers in a detailed field study of the four most common species *Geoica utricularia*, *Forda marginata*, *Forda formicaria* and *Tetraneura ulmi*. I showed that, in contrast to earlier records, all four species most likely reproduce clonally on Schiermonnikoog, although caution should be taken when excluding sexual reproduction for *T. ulmi* based on microsatellite data only (Chapter 3). The microsatellite data confirm that some aphid dispersal occurs along the island's salt marsh, but at very low frequencies. This indicates that sparse natural history records emanating from field surveys are not necessarily accurate, and that site specific life-history details of root aphids should ideally be verified prior to further experiments, especially when earlier records concern populations that have not been studied with genetic markers before.

Table 8.1 Overview of root aphid and mealybug species found in *Lasius flavus* nests at 13 field sites. For every location a description of the vegetation is given and the approximate search effort in days. Observed species are given for each site (grey cell). For species determination that remained uncertain, a question mark is given. No mealybugs were found or identified at sites with a minus sign. Mealybug species identification was performed by M. G. M. Jansen.

Location[1]	Vegetation	Search effort in days (appr.)	Aphids																Mealybugs				
			Anoecia corni	*Anoecia major*	*Anoecia pskovica*	*Anoecia zirnitsi*	*Aphis etiolata*	*Aphis striata*	*Baizongia pistaciae*	*Colopha compressa*	*Forda marginata*	*Forda formicara*	*Geoica utricularia*	*Geoica setulosa*	*Smynthurodes betae*	*Tetraneura ulmi*	*Trama troglodytes*		*Phenacoccus/Spinococcus?*	*Trionymus cf tomlini Green*	*Rhizoecus albidus Goux*	*Trionymus perrisii (Signoret)*	*Euripersia tomlini (Newstead)*
Junner Koeland , Ommen (NL)	Grazed floodplain grassland	7	■	■		■	■	■					■			■			–	–	–	–	–
Arrier Koeland , Ommen	Grazed floodplain grassland	3			■		■						■			■							■
Loc 1, Schiermonnikoog (NL)	Grazed salt marsh	15	■		■	■			■		■	■	■	■	■	■				■		■	
Loc 2, Schiermonnikoog,	Ungrazed salt marsh	45	■		?	■			?		■	■	■	■		■	?		■		■	■	
Groene Glop, Schiermonnikoog	Traditionally grazed pasture	2	■						?				■						–	–	–	–	–
Wassermann, Schiermonnikoog	Dune	2					■												–	–	–	–	–
Skallingen (DK)	Grazed salt marsh	1	■			■										■			–	–	–	–	–
Dejret (DK)	Grazed salt marsh	2	■	■	■	■							■						–	–	–	–	–
Ølst (DK)	Traditionally grazed pasture	1									?					?			–	–	–	–	–
Læsø (DK)	Grazed salt marsh	2				■				■	■	■	■			■	■		–	–	–	–	–
Holte (DK)	Traditionally grazed pasture	0.5									■								–	–	–	–	–
Dyrhavn (DK)	Abandoned hunting ground	0.5														■			–	–	–	–	–
Tiendeberg (B)	Chalk grassland	1	■			■													–	–	–	–	–

[1]At locations Møn (DK) and Höltigbaum (Ger.) only very few aphids were found. This is likely explained by suboptimal sampling conditions during the site visits.

In spite of some sampling efforts having remained limited (Table 8.1), the *L. flavus* populations across Northwest Europe may harbour an interesting degree of variation in aphid species diversity, their degree of myrmecophily, and possibly also reproductive modes and dispersal tendencies. This variation provides many additional opportunities to test alternative hypotheses on the implications of association with ants. This work would include population genetic analyses of aphids over their full ecological and geographical range, in particular including areas where aphid primary host plants occur and an ant-aphid conflict of interests over aphid reproductive mode is likely to be more pressing (Southern Europe for the focal species of Chapter 2 and 4). In addition, combined analyses of nuclear and mitochondrial DNA on sampled aphids from a wider geographical range, would allow construction of a phylogeny of aphid clonal lineages which will give more insight into the history of this mutualistic interaction and the origin of aphid clones found in Northwest Europe nowadays.

In particular the genus *Anoecia* offers a wide spectrum of these possibilities, with within-genus interspecific differences in reproductive mode, myrmecophily and dispersal tendencies (Paul 1977; Heie 1980). This means that in comparisons between *Anoecia* aphids, genus-differences in life-history can be factored out and yielded results may give more direct information on the evolutionary ecology of ant-tending than the aphid-aphid comparisons presented in this thesis. We already developed some microsatellite markers for this genus (Ivens *et al.* 2011; Chapter 5) and a preliminary taxonomic survey identified the possibility for several cryptic species in this species-complex (A.B.F. Ivens, personal observation), but time was too short to study this genus in detail.

What have we learned?

My empirical studies ended up focussing on the three most common aphid species. Chapter 2 and 3 show that *G. utricularia* and *F. marginata* reproduce exclusively clonally and *T. ulmi* most likely also. In addition dispersal is rare in all species. Ivens *et al.* (2012a; Chapter 4) then reveals that the majority of *L. flavus* nests contain only a single of these three species and that clonal lineages and species tend to occur spatially separated within ant mounds. Although the chapters presented in this thesis were largely aphid oriented, we also conducted several unpublished studies concerning the basic biology of the ants. Microsatellite analysis of ant workers collected from the same mounds as sampled for aphids, showed that the *L. flavus* population on Schiermonnikoog appears to consist of both mono- and polygynous colonies and that queens mate 1-2 times (Janzen 2009; van Boheemen 2010). Moreover, an ecological study that investigated correlations between ant colony genetic diversity, sex ratios and environmental stress factors such as inundation frequency (as approximated by salinity) and colony density showed, among other results, that ant sex ratios (males/females) are negatively correlated with salinity and that total reproductive output is negatively correlated with ant mound density (van Boheemen 2010; Koch 2012).

Our studies also gave further insights into incipient stages of the interaction, although additional work will be needed to fully resolve this. Our population genetic analyses on associated ants and aphids showed that host and symbiont population structures differ strongly, with aphid populations being highly structured, and the ant population being

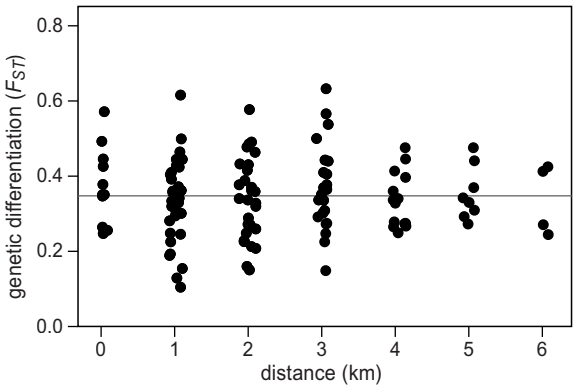


Figure 8.3 Lack of correlation between genetic differentiation and geographic distance between sample locations of the ant *Lasius flavus* on Schiermonnikoog. Genetic differentiation is quantified as F_{ST} (Weir & Cockerham 1984). The dashed line shows the linear regression line between genetic differentiation and distance indicating that no significant correlation (Mantel test: N = 120, P = 0.486) was observed between locations along the 7 km transect (figure reproduced from Janzen 2009).

well-mixed, i.e. panmictic. In fact, the ant population does not show any population differentiation, neither along the sampled transect (Figure 8.3), nor across Northwestern Europe (Janzen 2009). *L. niger* is also known to have a such well-mixed population, which is consistent with both *Lasius* species mating in large, synchronized mating flights (Boomsma & Van der Have 1998). This absence of genetic correlation between ants and aphids indicates that vertical transmission can be excluded as transmission mode for the aphids. In addition, virgin queens have never been observed to carry aphids on their mating flight, another indication that vertical transmission does not occur [there are examples of dispersing virgin queens carrying symbiotic coccids; (Hölldobler & Wilson 1990)]. Aphids thus transmit horizontally between ant colonies and several mechanisms can underlie aphid this form of transmission: horizontally dispersing aphids can enter existing ant colonies and/or be picked up by ants; this mechanism is supported by the finding that ants are able to discriminate between nymphs and adults, but do often not seem to be able to distinguish between aphid species or clones (Box A). Alternatively, groups of aphids may remain in the ant mound, being tended by subsequent colonies of ants. Determining the relative importance of these two scenarios for horizontal transmission will require more detailed studies on ant-aphid partner choice and long-term monitoring of existing and incipient ant colonies.

The big unknowns, however, remain the costs and benefits for both host and symbionts of being engaged in their interaction. The benefits to the ants are presumed to lie in aphids providing both carbohydrates and proteins. The aphids are presumed to not survive without the ants, as is confirmed by my own observations. In addition, Smart (1991) showed that aphid population size increases with ant attendance. Thus, while it seems beyond doubt that both host and symbionts benefit from the interaction, the relationships between costs and benefits of symbiosis and their respective effects on survival and repro-

ductive success would need to be quantified before we can draw firmer conclusion on the forces that affect the dynamics of this mutualism.

Scope for the *L. flavus* – root aphid system as a future model system for farming mutualism?

Does the *Lasius flavus* – root aphid system have the potential to become a new model system for farming mutualisms? In order to understand universal factors governing the evolutionary ecology of farming mutualisms, we need systems that are similar enough to allow meaningful comparison, yet different enough to highlight the importance of system-specific characteristics. The best known farming mutualisms are those of social insects farming crops: the fungus-growing ants and termites. Just like these systems, the *L. flavus* system is presumed to be an obligate mutualism with in-nest farming of symbionts, unlike other examples of 'cattle-breeding' ants such as *L. niger* that facultatively tend above-ground aphids. For a further understanding of (obligate) farming mutualisms, having a study system at hand such as *L. flavus* is therefore indispensable. Moreover, the fact that both *Lasius* species sometimes tend the same aphid species opens doors to studying mutualism networks. Likewise, promising future studies of this mutualism can extend to including aphid host plants (Veen 2011) and aphid endosymbionts.

My studies showed that population genetic field studies are feasible and that the system has a lot of research potential in terms of mapping aphid species diversity and life-history variation across mounds and (sub)populations. Controlled laboratory studies, however, will remain a challenge in this subterranean system, with observations remaining restricted to artificial environments (as used by Muir 1960; Dixon 1970; Paul 1977; Smart 1991) with low aphid numbers. In addition, the interpretation of experimental data will be difficult as long as basic biological information, such as how partners associate, is not well understood. Developing this system into a model system would thus first require a large number of descriptive studies, before conclusive experimental data can be obtained. In fact, these descriptive studies might constitute the biggest hurdle towards adopting this system as a model system: in the current scientific culture of publish-or-perish, purely descriptive, often time-consuming, studies have a hard time to be published in the highest ranking journals. Nevertheless, I think that it is worth taking this hurdle, because few other farming mutualisms have that much potential for studying the many facets of in-house cattle breeding in a single system.

FARMING MUTUALISMS

Farming mutualisms are interactions between different species in which one species (the host) promotes the growth of a species it relies on for food (the symbiont). Farming interactions are considered to be *mutualisms* when they are presumed to yield net benefits for both partners. In general, the exchanged benefits in farming interactions are nutrients provided by the symbiont (either in the form of goods produced, or by being eaten or both) in exchange for host protection and tending so their life-time reproductive success is enhanced. In asymmetric interactions in which the host potentially controls the symbiont,

host-symbiont conflicts of interests are expected. The three main arenas of potential conflict are (1) symbiont reproductive mode, (2) symbiont transmission and dispersal and (3) symbiont diversity (see also Chapter 1).

Universal patterns in farming mutualisms?

For each of the three potential arenas of conflict, generally two alternative outcomes in terms of symbiont traits are considered possible: (1) symbiont reproduction can be sexual or asexual, (2) symbiont transmission can be vertical or horizontal, and (3) symbiont diversity can be minimal (monoculture – clonal relatedness of 100%) or substantial (polyculture – relatedness moderate to low) (Herre *et al.* 1999; Mueller 2002; Table 1.2). Host and symbiont may prefer different outcomes and hence a conflict of interest can arise. In this thesis, I studied which combination of outcomes characterizes the mutualistic interaction between ant *L. flavus* and the root aphids in its nest. I showed that in this system, symbiont reproduction is asexual in three of the studied species and also likely asexual in the fourth, transmission is horizontal (and rare), and symbiont diversity is low, especially at the lowest organization-level of aphid chambers. This particular combination of outcomes is not commonly observed in other farming mutualisms (see Figure 1.1). Other farmed obligate symbionts are often asexually vertically transmitted and kept in genetic monocultures [*e.g.* fungus-growing ants; some fungus-growing termites (Weber 1972; Korb & Aanen 2003; Mueller *et al.* 2005; Poulsen & Boomsma 2005; Mueller *et al.* 2010)] or symbionts reproduce sexually and transmit horizontally, after which diversity remains or is actively decreased by hosts [above-ground aphids (facultatively) kept by ants; the majority of fungus-growing termites; algae-farming damselfish (Hata & Kato 2004; Aanen *et al.* 2009; Yao 2010)].

In the other obligate systems of fungus-farming insects, symbiont reproduction, transmission and diversity are controlled by an interplay of several host and symbiont control mechanisms (a.o. Bot *et al.* 2001; Poulsen & Boomsma 2005; Aanen *et al.* 2009; Poulsen *et al.* 2009; Schiøtt *et al.* 2010). In the *L. flavus* – system, however, the underlying mechanisms governing these symbiont traits remain unclear. Does our observed set of outcomes result from *L. flavus* host control over symbiont dispersal, reproduction and diversity? Or do the strong aphid population structure and compartmentalization of aphid clones within ant mounds simply result from constraints on aphid dispersal and does the absence of sexual reproduction in aphids result from the absence of suitable host plants? Whether active host control mechanisms or symbiont dispersal constraints underlie our findings remains unclear. We can, however, confirm based on our data in combination with previous findings in other farming mutualisms (Bot *et al.* 2001; Hata & Kato 2002; Poulsen 2005; Aanen *et al.* 2009; Mueller *et al.* 2010), that many of the farming systems that have been stable over evolutionary time are characterized by low symbiont diversity at the lowest organization level. Often this low diversity goes hand in hand with asexual reproduction and vertical transmission. My results show that also with horizontal transmission low diversity can be achieved, be it actively or passively (see also Aanen *et al.* 2009). Care should be taken when comparing obligate to facultative systems and systems with host control to systems without host control, because very different mechanisms may be at work. Nevertheless, recognizing universal patterns in terms of identical outcomes regardless of

different underlying mechanisms may give hints as to what makes mutualistic systems robust against cheating so they do not fall victim to mutualism breakdown (Sachs & Simms 2006). Low diversity through compartmentalization might be one of these universal, stabilizing factors (Frank 1996; Herre *et al.* 1999), but see Verbruggen *et al.* (2012) who show that spatial structure can also hamper the stability of mutualism in mycorrhiza.

Future avenues for modelling (farming) mutualism

In sum, a more complete understanding of the evolutionary ecology of mutualisms requires both empirical studies on a wider array of farming mutualisms and theoretical modelling effort specifically tailored to farming mutualism. In this section I would like to describe how a suitable model for the evolution of farming mutualism would look like, thereby taking into consideration the empirical and theoretical findings on farming mutualism I discussed in the two previous sections.

In my opinion, more encompassing models for the evolution of farming mutualism should include the following features:

- *Asymmetric host-symbiont interaction* (in terms of cost-benefit ratio, goods or services exchanged, generation time, body size, number of individuals): asymmetry on one or more levels is a key characteristic of farming mutualism
- *Nutritional benefit for the host*: by definition, the host is dependent on the symbiont for food, and the condition of the host should thus directly improve with consuming (part of) the symbiont.
- *Protection and uniform environment provided by the host*: in many farming mutualisms, host protection increases symbiont survival and decreases environmental fluctuations, by creating a uniform environment.
- *Different symbiont reproductive modes possible*: sexual vs. asexual: the two alternative outcomes of the conflict on reproductive mode
- *Different symbiont transmission modes possible*: vertical vs. horizontal: the two alternative outcomes for the conflict on transmission mode
- *Different levels of symbiont diversity possible*: monoculture vs. different degrees of polyculture.
- *Host control over symbiont reproduction, transmission and diversity (symbiont relatedness) should be allowed to evolve*: host control is often assumed to be the driving force behind conflict resolution in farming mutualism. However, host control should not be an *a priori* assumption but an evolving trait, as it will undergo evolutionary change when the symbiosis becomes more integrated.
- *Co-transmission and simultaneous timing of reproduction should be allowed to undergo evolutionary change*: these two mechanisms can lead to positive assortment of mutualistic partners. Implementing these mechanisms can help evaluating the importance of positive assortment for the evolution of mutualism.

In addition, my thesis results indicate that a good model for mutualism in general should ideally include the following two components: (1) conditional behaviour of the interacting partners and (2) evolving cost-benefit ratios. First, conditional behaviour allows for higher

degrees of freedom for interacting partners, which can lead to unforeseen co-evolutionary dynamics that may remain overlooked when behaviour is presumed to be unconditional in a model (Chapter 6, 7). Second, allowing evolution of variables that have hitherto been considered constant can also give new insights into mutualism dynamics. Joint evolution of mutualism intensity and cost-benefits ratios is particularly important, because it allows modelled mutualisms to move along the mutualism-parasitism continuum.

A model with these features would allow for an in-depth analysis of the outcomes of host-symbiont conflict and the mechanisms governing them in specific biological systems. For example, is low symbiont diversity indeed a basic requirement for stable farming mutualism to evolve, as comparison of natural farming mutualisms suggests? And what is the relative importance of mechanisms like host control, partner fidelity and partner choice? Is each of these necessary for stable farming mutualism, or can mutualism remain stable also in the absence of one of them? Lastly, such a model would give us insight in the mutualism-parasitism dynamics of farming mutualisms.

CONCLUSION

The establishment of a mutually beneficial relationship requires partners to be able to repeatedly choose to continue an interaction (partner fidelity). Although most farming mutualisms may start under this condition, subsequent co-evolution, inherent asymmetry, and potential conflicts of interests will ultimately erode the initial degrees of freedom of the partners in terms of dispersal, reproduction and partner choice. However, giving up on individual freedom may be compensated for by being part of an increasingly productive, specialized system in which the individual partners are more successful than they would have been on their own. This is also what likely happened to the root aphids tended by Yellow meadow ants: the protective environment provided by the ants might have given the aphids the opportunity to give up almost all sexual reproduction, stay at the same location for prolonged periods of time and spread northwards from their original habitat. Vice versa, the year-round presence of aphids for 'milk and meat' gives the ants the opportunity to reach higher population densities than they could reach in salt marsh habitats without aphids. It is therefore safe to say that, for these ants and aphids, life seems better together.

Why are we here? And where do we go? And how come it's so hard?
It's not always easy and sometimes life can be deceiving
I'll tell you one thing, it's always better when we're together

- Better Together, Jack Johnson (2004) -

References

Aanen DK, De Fine Licht HH, Debets AJM, Kerstes NAG, Hoekstra RF, Boomsma JJ (2009) High symbiont relatedness stabilizes mutualistic cooperation in fungus-growing termites. *Science*, **326**, 1103-1106.

Aanen DK, Eggleton P, Rouland-Lefevre C, Guldberg-Froslev T, Rosendahl S, Boomsma JJ (2002) The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14887-14892.

Aanen DK, Hoekstra RF (2007) The evolution of obligate mutualism: if you can't beat 'em, join 'em. *Trends in Ecology & Evolution*, **22**, 506-509.

Abbot P (2009) On the evolution of dispersal and altruism in aphids. *Evolution*, **63**, 2687-2696.

Abbot P, Abe J, Alcock J, et al (2011) Inclusive fitness theory and eusociality. *Nature*, **471**, E1-E4.

Abbot P, Chhatre V (2007) Kin structure provides no explanation for intruders in social aphids. *Molecular Ecology*, **16**, 3659-3670.

Allen DE, Lynch M (2012) The effect of variable frequency of sexual reproduction on the genetic structure of natural populations of a cyclical parthenogen. *Evolution*, **66**, 919-926.

Archetti M, Úbeda F, Fudenberg D, Green J, Pierce NE, Yu DW (2011) Let the right one in: a microeconomic approach to partner choice in mutualisms. *The American Naturalist*, **177**, 75-85.

Arnaud-Haond S, Duarte CM, Alberto F, Serrao EA (2007) Standardizing methods to address clonality in population studies. *Molecular Ecology*, **16**, 5115-5139.

Arnold A, Herre E (2003) Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia*, **95**, 388-398.

Axelrod R, Hamilton WD (1981) The evolution of cooperation. *Science*, **211**, 1390-1396.

B

Bäckhed F, Ley R, Sonnenburg J, Peterson D, Gordon J (2005) Host-bacterial mutualism in the human intestine. *Science*, **307**, 1915-1920.

Balloux F, Lehmann L, De Meeus T (2003) The population genetics of clonal and partially clonal diploids. *Genetics*, **164**, 1635-1644.

Bascompte J, Jordano P, Olesen JM (2006) Asymmetric coevolutionary networks facilitate biodiversity maintenance. *Science*, **312**, 431-433.

Bastolla U, Fortuna MA, Pascual-Garcia A, Ferrera A, Luque B, Bascompte J (2009) The architecture of mutualistic networks minimizes competition and increases biodiversity. *Nature*, **458**, 1018-U91.

Ben-Dov Y, Fischer BL (2010) The mutualism of *Melissotarsus* ants and armoured scale insects in Africa and Madagascar: distribution, host plants and biology. *Entomologica Hellenica*, **19**, 45-53.

Bengtsson B (2003) Genetic variation in organisms with sexual and asexual reproduction. *Journal of Evolutionary Biology*, **16**, 189-199.

Bergstorm CT, Bronstein JL, Bshary R, et al (2002) Interspecific mutualism - Puzzels and predictions. In: *Genetic and cultural evolution of cooperation* pp. 241-257 The MIT Press, Cambridge, Massachusetts.

Bever JD, Richardson SC, Lawrence BM, Holmes J, Watson M (2009) Preferential allocation to beneficial symbiont with spatial structure maintains mycorrhizal mutualism. *Ecology Letters*, **12**, 13-21.

Biedermann PHW, Taborsky M (2011) Larval helpers and age polyethism in ambrosia beetles. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 17064-17069.

Bijma P, Aanen DK (2010) Assortment, Hamilton's rule and multilevel selection. *Proceedings of the Royal Society B-Biological Sciences*, **277**, 673-675.

Birky CW (1996) Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics*, **144**, 427-437.

Blackman RL, Eastop VF (1994) *Aphids on the world's trees - An identification and Information Guide* CAB International, Oxon, UK.

Blomqvist MM, Olff H, Blaauw MB, Bongers T, van der Putten WH (2000) Interactions between above- and belowground biota: importance for small-scale vegetation mosaics in a grassland ecosystem. *Oikos*, **90**, 582-598.

Booijink CCGM, Zoetendal EG, Kleerebezem M, de Vos WM (2007) Microbial communities in the human small intestine: coupling diversity to metagenomics. *Future Microbiology*, **2**, 285-295.

Boomsma JJ, Isaaks JA (1982) Effects of inundation and salt on the survival of ants in a sandy coastal plain *Ecological Entomology*, **7**, 121-130.
Boomsma JJ, Van loon AJ (1982) Structure and diversity of ant communities in successive coastal dune valleys. *Journal of Animal Ecology*, **51**, 957-974.
Boomsma JJ, Wright PJ, Brouwer AH (1993) Social structure in the ant *Lasius flavus* - Multi-queen nests or multi-nest mounds. *Ecological Entomology*, **18**, 47-53.
Boomsma JJ, Beekman M, Cornwallis CK, et al (2011) Only full-sibling families evolved eusociality. *Nature*, **471**, E4-E5.
Boomsma J, Van der Have T (1998) Queen mating and paternity variation in the ant *Lasius niger*. *Molecular Ecology*, **7**, 1709-1718.
Bot ANM, Rehner SA, Boomsma JJ (2001) Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. *Evolution*, **55**, 1980-1991.
Botero CA, Pen I, Komdeur J, Weissing FJ (2010) The evolution of individual variation in communication strategies. *Evolution*, **64**, 3123-3133.
Bourke AFG (2011) *Principles of social evolution*. Oxford University Press Inc., New York.
Brock DA, Douglas TE, Queller DC, Strassmann JE (2011) Primitive agriculture in a social amoeba. *Nature*, **469**, 393-396.
Bronstein JL (2001) The exploitation of mutualisms. *Ecology Letters*, **4**, 277-287.
Bronstein JL (1994) Conditional Outcomes in Mutualistic Interactions. *Trends in Ecology & Evolution*, **9**, 214-217.
Brown JL (1983) Cooperation - A biologists dilemma. *Advances in the Study of Behaviour*, **13**, 1-37.
Bshary R, Grutter AS (2002) Asymmetric cheating opportunities and partner control in a cleaner fish mutualism. *Animal Behaviour*, **63**, 547-555.
Bshary R, Noë R, Hammerstein P (2002) Biological markets: The ubiquitous influence of partner choice on the dynamics of cleaner fish-client reef fish interactions. In: *Genetic and cultural evolution of cooperation*. pp. 167-185 The MIT Press, Cambridge, Massachusetts, London, England.
Bshary R, Grutter AS (2006) Image scoring and cooperation in a cleaner fish mutualism. *Nature*, **441**, 975-978.
Bull JJ, Rice WR (1991) Distinguishing mechanisms for the evolution of cooperation. *Journal of Theoretical Biology*, **149**, 63-74.

C

Connor RC (1995) The benefits of mutualism - a conceptual framework. *Biological reviews of the Cambridge Philosophical Society*, **70**, 427-457.

D

Darwin C (1859) Difficulties on theory. In: *On the origin of species*, 2004 edn, pp. 191-228 Collector's Library, London.
Dawkins R (1976) *The selfish gene.* Oxford University Press, Oxford.
De Meeus T, Balloux F (2004) Clonal reproduction and linkage disequilibrium in diploids: a simulation study. *Infection, Genetics and Evolution*, **4**, 345-351.
De Meeus T, Lehmann L, Balloux F (2006) Molecular epidemiology of clonal diploids: A quick overview and a short DIY (do it yourself) notice. *Infection, Genetics and Evolution*, **6**, 163-170.
De Meeus T, Prugnolle F, Agnew P (2007) Asexual reproduction: Genetics and evolutionary aspects. *Cellular and Molecular Life Sciences*, **64**, 1355-1372.
Dean WRJ, Milton SJ, Klotz S (1997) The role of ant nest-mounds in maintaining small-scale patchiness in dry grasslands in Central Germany. *Biodiversity and Conservation*, **6**, 1293-1307.
Delmotte F, Leterme N, Gauthier JP, Rispe C, Simon JC (2002) Genetic architecture of sexual and asexual populations of the aphid *Rhopalosiphum padi* based on allozyme and microsatellite markers. *Molecular Ecology*, **11**, 711-723.
Dixon AFG (1970) Aphids as root-fluid feeders. In: *Methods of study in soil ecology* (ed. Philopson J) pp. 243-247 UNESCO, Paris.
Doebeli M, Knowlton N (1998) The evolution of interspecific mutualisms. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 8676-8680.

Dorken ME, Eckert CG (2001) Severely reduced sexual reproduction in northern populations of a clonal plant, *Decodon verticillatus* (Lythraceae). *Journal of Ecology*, **89**, 339-350.
D'Souza TG, Michiels NK (2010) The costs and benefits of occasional sex: theoretical predictions and a case study. *Journal of Heredity*, **101**, S34-S41.
D'Souza TG, Michiels NK (2006) Genetic signatures of occasional sex in parthenogenetic subpopulations of the freshwater planarian *Schmidtea polychroa*. *Freshwater Biology*, **51**, 1890-1900.
E
Edwards DP, Hassall M, Sutherland WJ, Yu DW (2006) Selection for protection in an ant-plant mutualism: host sanctions, host modularity and the principal-agent game. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 595-602.
Ellstrand NC, Roose ML (1987) Patterns of genotypic diversity in clonal plant-species. *American Journal of Botany*, **74**, 123-131.
F
Farrell BD, Sequeira AS, O'Meara BC, Normark BB, Chung JH, Jordal BH (2001) The evolution of agriculture in beetles (Curculionidae : Scolytinae and Platypodinae). *Evolution*, **55**, 2011-2027.
Fawcett TW, Kuijper B, Weissing FJ, Pen I (2011) Sex-ratio control erodes sexual selection, revealing evolutionary feedback from adaptive plasticity. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 15925-15930.
Ferrière R, Bronstein JL, Rinaldi S, Law R, Gauduchon M (2002) Cheating and the evolutionary stability of mutualisms. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 773-780.
Ferrière R, Michod RE (2011) Inclusive fitness in evolution. *Nature*, **471**, E6-E8.
Firth D (1993) Bias reduction of maximum likelihood estimates. *Biometrica*, **80**, 27-38.
Fischer MK, Shingleton AW (2001) Host plant and ants influence the honeydew sugar composition of aphids. *Functional Ecology*, **15**, 544-550.
Fletcher JA, Doebeli M (2009) A simple and general explanation for the evolution of altruism. *Proceedings of the Royal Society B-Biological Sciences*, **276**, 13-19.
Foster KR, Kokko H (2006) Cheating can stabilize cooperation in mutualisms. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 2233-2239.
Foster KR, Wenseleers T (2006) A general model for the evolution of mutualisms. *Journal of Evolutionary Biology*, **19**, 1283-1293.
Foster WA (1978) Dispersal behaviour of an intertidal aphid. *Journal of Animal Ecology*, **47**, 653-659.
Foster WA (1975) Life-history and population biology of an intertidal aphid, *Pemphigus trehernei* Foster. *Transactions of the Royal Entomological Society of London*, **127**, 193-207.
Foster WA, Treherne JE (1978) Dispersal mechanisms in an intertidal aphid. *Journal of Animal Ecology*, **47**, 205-217.
Frank SA (1996) Host-symbiont conflict over the mixing of symbiotic lineages. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **263**, 339-344.
Frank SA (1994) Genetics of mutualism - the evolution of altruism between species. *Journal of Theoretical Biology*, **170**, 393-400.
Fu F, Hauert C, Nowak MA, Wang L (2008) Reputation-based partner choice promotes cooperation in social networks. *Physical Review E*, **78**, 026117.
G
Gilabert A, Simon JC, Mieuzet L, et al (2009) Climate and agricultural context shape reproductive mode variation in an aphid crop pest. *Molecular Ecology*, **18**, 3050-3061.
Giske M, Eliassen S, Fisken Ø, et al (in press) Maintenance of genotypic and phenotypic variation in architecture, emotions and behaviour. *Proceedings of the National Academy of Sciences of the United States of America*, .
Godske L (1992) Aphids in nests of *Lasius flavus* F. in Denmark II: Population dynamics. *Entomologiske Meddelelser*, **60**, 21-26.
Godske L (1991) Aphids in nests of *Lasius flavus* F. in Denmark I: Faunistic description. *Entomologiske Meddelelser*, **59**, 85-89.

Gorospe KD, Karl SA (in review) Genetic relatedness does not retain spatial pattern across multiple spatial scales: dispersal and colonization in the coral, *Pocillopora damicornis*. *PloS One*.
Green RF, Noakes DLG (1995) Is a little bit of sex as good as a lot? *Journal of Theoretical Biology*, **174**, 87-96.

H

Hales DF, Tomiuk J, Wohrmann K, Sunnucks P (1997) Evolutionary and genetic aspects of aphid biology: A review. *European Journal of Entomology*, **94**, 1-55.
Halkett F, Simon J, Balloux F (2005) Tackling the population genetics of clonal and partially clonal organisms. *Trends in Ecology & Evolution*, **20**, 194-201.
Hallatschek O, Hersen P, Ramanathan S, Nelson DR (2007) Genetic drift at expanding frontiers promotes gene segregation. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 19926-19930.
Hamilton WD (1964a) Genetical evolution of social behaviour 1. *Journal of Theoretical Biology*, **7**, 1-16.
Hamilton WD (1964b) Genetical evolution of social behaviour 2. *Journal of Theoretical Biology*, **7**, 17-52.
Hamilton WD, May RM (1977) Dispersal in stable habitats. *Nature*, **269**, 578-581.
Harper J (1977) *Population biology of plants.* Academic Press, London.
Hartl DL, Clark AG (1997) *Principles of population genetics.* Sunderland, Sinauer Ass.
Hata H, Kato M (2006) A novel obligate cultivation mutualism between damselfish and Polysiphonia algae. *Biology Letters*, **2**, 593-596.
Hata H, Kato M (2004) Monoculture and mixed-species algal farms on a coral reef are maintained through intensive and extensive management by damselfishes. *Journal of experimental marine biology and ecology*, **313**, 285-296.
Hata H, Kato M (2002) Weeding by the herbivorous damselfish *Stegastes nigricans* in nearly monocultural algae farms. *Marine Ecology-Progress Series*, **237**, 227-231.
Hata H, Watanabe K, Kato M (2010) Geographic variation in the damselfish-red alga cultivation mutualism in the Indo-West Pacific. *BMC Evolutionary Biology*, **10**.
Heie OE (1986) The Aphidoidea (Hemiptera) of Fennoscandia and Denmark. III. *Fauna Entomologica Scandinavica*, **17**, 1-314.
Heie OE (1980) The Aphidoidea (Hemiptera) of Fennoscandia and Denmark. I. *Fauna Entomologica Scandinavica*, **9**, 1-236.
Herre EA, Knowlton N, Mueller UG, Rehner SA (1999) The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution*, **14**, 49-53.
Hoeksema JD, Bruna EM (2000) Pursuing the big questions about interspecific mutualism: a review of theoretical approaches. *Oecologia*, **125**, 321-330.
Hölldobler B, Wilson EO (1990) *The Ants.* Harvard University Press, Cambridge,MA.
Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. *Biometrical Journal*, **50**, 346-363.
Hruschka D, Henrich J (2006) Friendship, cliquishness, and the emergence of cooperation. *Journal of Theoretical Biology*, **239**, 1-15.
Hurst L, Peck J (1996) Recent advances in understanding of the evolution and maintenance of sex. *Trends in Ecology & Evolution*, **11**, A46-A52.

I

Ichinose G, Arita T (2008) The role of migration and founder effect for the evolution of cooperation in a multilevel selection context. *Ecological Modelling*, **210**, 221-230.
Ivens ABF, Kronauer DJC, Pen I, Weissing FJ, Boomsma JJ (2012a) Ants farm subterranean aphids mostly in single clone groups – an example of prudent husbandry for carbohydrates and proteins? *BMC Evolutionary Biology*, **12**, 106.
Ivens ABF, Kronauer DJC, Pen I, Weissing FJ, Boomsma JJ (2012b) Reproduction and dispersal in an ant-associated root aphid community. *Molecular Ecology*, **21**, 4257-4269.
Ivens ABF, Kronauer DJC, Boomsma JJ (2011) Characterisation and cross-amplification of polymorphic microsatellite loci in ant-associated root-aphids. *Conservation Genetics Resources*, **3**, 73-77.

Ivens ABF, Nash DR, Poulsen M, Boomsma JJ (2009) Caste-specific symbiont policing by workers of *Acromyrmex* fungus-growing ants. *Behavioural Ecology*, **20**, 378-384.

J

James F (1994) RANLUX: A Fortran implementation of the high-quality pseudo-random number generator of Lüscher. *Computer Physics Communications*, **79**, 111-114.

Jander KC, Herre EA (2010) Host sanctions and pollinator cheating in the fig tree-fig wasp mutualism. *Proceedings of the Royal Society B-Biological Sciences*, **277**, 1481-1488.

Jansen VAA, van Baalen M (2006) Altruism through beard chromodynamics. *Nature*, **440**, 663-666.

Janssen MA, Goldstone RL (2006) Dynamic-persistence of cooperation in public good games when group size is dynamic. *Journal of Theoretical Biology*, **243**, 134-142.

Janzen T (2009) *Within nest genetics of Lasius flavus and one of its mutualists* Tetraneura ulmi. M.Sc. Thesis, University of Groningen, Groningen.

Jin L, Chakraborty R (1993) A bias-corrected estimate of heterozygosity for single-probe multilocus DNA fingerprints. *Molecular Biology and Evolution*, **10**, 1112-1114.

Johnson PCD, Llewellyn KS, Amos W (2000) Microsatellite loci for studying clonal mixing, population structure and inbreeding in a social aphid, Pemphigus spyrothecae (Hemiptera : Pemphigidae). *Molecular Ecology*, **9**, 1445-1446.

K

Kanbe T, Akimoto SI (2009) Allelic and genotypic diversity in long-term asexual populations of the pea aphid, *Acyrthosiphon pisum* in comparison with sexual populations. *Molecular Ecology*, **18**, 801-816.

Keller L, Ross KG (1998) Selfish genes: a green beard in the red fire ant. *Nature*, **394**, 573-575.

Kiers ET, Denison RF (2008) Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual Review of Ecology Evolution and Systematics*, **39**, 215-236.

Kiers ET, Denison RF, Kawakita A, Herre EA (2011) The biological reality of host sanctions and partner fidelity. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, E7-E7.

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. *Nature*, **425**, 78-81.

Killingback T, Bieri J, Flatt T (2006) Evolution in group-structured populations can resolve the tragedy of the commons. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 1477-1481.

Kindlmann P, Hulle M, Stadler B (2007) Timing of dispersal: effect of ants on aphids. *Oecologia*, **152**, 625-631.

Koch W (2012) *Effects of intra-colony relatedness and environmental stress on the production of sexuals in the ant* Lasius flavus. M.Sc. Thesis, University of Groningen, Groningen.

Korb J, Aanen DK (2003) The evolution of uniparental transmission of fungal symbionts in fungus-growing termites (Macrotermitinae). *Behavioural Ecology and Sociobiology*, **53**, 65-71.

Kosztarab M, Kozár F (1988) *Scale insects of Central Europe.* Akademiai Kiado, Budapest, Hungary.

Kronauer DJC, Pierce NE, Keller L (in review) Invasion of the clones – The global spread of the parthenogenetic ant Cerapachys biroi. *Molecular Ecology*.

Kümmerli R, Gardner A, West SA, Griffin AS (2009) Limited dispersal, budding dispersal, and cooperation: an experimental study. *Evolution*, **63**, 939-949.

L

Laland KN, Boogert NJ (2010) Niche construction, co-evolution and biodiversity. *Ecological Economics*, **69**, 731-736.

Larsen CS (1995) Biological changes in human populations with agriculture. *Annual Review of Anthropology*, **24**, 185-213.

Law R (1985) Evolution in a mutualistic environment. In: *The Biology of Mutualism* (ed. Boucher DH) pp. 145-191 Oxford University Press, Oxford.

Law R, Lewis DH (1983) Biotic environments and the maintenance of sex - some evidence from mutualistic symbioses. *Biological Journal of the Linnean Society*, **20**, 249-276.

Le Galliard JF, Ferrière R, Dieckmann U (2003) The adaptive dynamics of altruism in spatially heterogeneous populations. *Evolution*, **57**, 1-17.

Lehmann L, Keller L (2006) The evolution of cooperation and altruism - a general framework and a classification of models. *Journal of Evolutionary Biology*, **19**, 1365-1376.
Leigh EG (2010) The evolution of mutualism. *Journal of Evolutionary Biology*, **23**, 2507-2528.
Leimar O, Connor RC (2003) By-product benefits, reciprocity and pseudoreciprocity in mutualism. In: *Genetic and cultural evolution of cooperation* (ed. Hammerstein P) pp. 203-222 The MIT press, Cambridge, MA.
Lenoir L (2009) Effects of ants on plant diversity in semi-natural grasslands. *Arthropod-Plant Interactions*, **3**, 163-172.
Login FH, Balmand S, Vallier A, *et al.* (2011) Antimicrobial peptides keep insect endosymbionts under control. *Science*, **334**, 362-365.
Lüscher M (1994) A portable high-quality random number generator for lattice field theory calculations. *Computer Physics Communications*, **79**, 100-110.

M

Mack KML (2012) Selective feedback between dispersal distance and the stability of mutualism. *Oikos*, **121**, 442-448.
Margulis L (1971) Symbiosis and evolution. *Scientific American*, **225**, 49-55.
McFall-Ngai M (2008) Hawaiian bobtail squid. *Current Biology*, **18**, R1043-R1044.
McNamara JM, Barta Z, Fromhage L, Houston AI (2008) The coevolution of choosiness and cooperation. *Nature*, **451**, 189-192.
Michel AP, Zhang W, Jung JK, Kang S, Mian MAR (2009) Population genetic structure of *Aphis glycines*. *Environmental Entomology*, **38**, 1301-1311.
Mikheyev AS, Mueller UG, Abbot P (2006) Cryptic sex and many-to-one colevolution in the fungus-growing ant symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 10702-10706.
Miller NJ, Birley AJ, Tatchell GM (2000) Polymorphic microsatellite loci from the lettuce root aphid, *Pemphigus bursarius*. *Molecular Ecology*, **9**, 1951-1952.
Moran NA (1992) The evolution of aphid life-cycles. *Annual Review of Entomology*, **37**, 321-348.
Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, **42**, 165-190.
Mueller UG (2002) Ant versus fungus versus mutualism: Ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. *American Naturalist*, **160**, S67-S98.
Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR (2005) The evolution of agriculture in insects. *Annual Review of Ecology Evolution and Systematics*, **36**, 563-595.
Mueller UG, Rehner SA, Schultz TR (1998) The evolution of agriculture in ants. *Science*, **281**, 2034-2038.
Mueller UG, Scott JJ, Ishak HD, Cooper M, Rodrigues A (2010) Monoculture of leafcutter ant gardens. *Plos One*, **5**, e12668.
Muir DA (1960) A culture method for myrmecophilous root aphids. *Bulletin of Entomological Research*, **51**, 7-8.
Muir DA (1959) The ant-aphid-plant relationship in West Dunbartonshire. *Journal of Animal Ecology*, **28**, 133-140.
Mylius S, Diekmann O (1995) On evolutionarily stable life histories, optimization and the need to be specific about density dependence. *Oikos*, **74**, 218-224.

N

Nadell CD, Foster KR, Xavier JB (2010) Emergence of spatial structure in cell groups and the evolution of cooperation. *PLoS computational biology*, **6**, e1000716.
Nauta MJ, Weissing FJ (1996) Constraints on allele size at microsatellite loci: implications for genetic differentiation. *Genetics*, **143**, 1021-1032.
Noë R, Hammerstein P (1994) Biological markets - supply and demand determine the effect of partner choice in cooperation, mutualism and mating. *Behavioural Ecology and Sociobiology*, **35**, 1-11.
Nowak M, Sigmund K (1993) A strategy of win-stay, lose-shift that outperforms Tit-For-Tat in the Prisoners Dilemma game. *Nature*, **364**, 56-58.

Nowak MA (2006) Five rules for the evolution of cooperation. *Science*, **314**, 1560-1563.
Nowak MA, May RM (1992) Evolutionary games and spatial chaos. *Nature*, **359**, 826-829.
Nowak MA, Tarnita CE, Wilson EO (2011) In defence of inclusive fitness theory. *Nature*, **471**, E9-E10.
Nowak MA, Tarnita CE, Wilson EO (2010) The evolution of eusociality. *Nature*, **466**, 1057-1062.

O

Odum EP, Pontin AJ (1961) Population density of underground ant, *Lasius Flavus*, as determined by tagging with P-32. *Ecology*, **42**, 186-188.
Offenberg J (2001) Balancing between mutualism and exploitation: the symbiotic interaction between *Lasius* ants and aphids. *Behavioural Ecology and Sociobiology*, **49**, 304-310.
Ohtsuki H, Hauert C, Lieberman E, Nowak M (2006) A simple rule for the evolution of cooperation on graphs and social networks. *Nature*, **441**, 502-505.
Olff H, De Leeuw J, Bakker J, Platerink R, Van Wijnen H, De Munck W (1997) Vegetation succession and herbivory in a salt marsh: changes induced by sea level rise and silt deposition along an elevational gradient. *Journal of Ecology*, **85**, 799-814.
Oliver TH, Leather SR, Cook JM (2009) Tolerance traits and the stability of mutualism. *Oikos*, **118**, 346-352.
Oliver TH, Mashanova A, Leather SR, Cook JM, Jansen VAA (2007) Ant semiochemicals limit apterous aphid dispersal. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 3127-3131.

P

Palfner G, Casanova-Katny M, Read D (2005) The mycorrhizal community in a forest chronosequence of Sitka spruce [*Picea sitchensis* (Bong.) Carr.] in Northern England. *Mycorrhiza*, **15**, 571-579.
Palmer TM, Doak DF, Stanton ML, et al (2010) Synergy of multiple partners, including freeloaders, increases host fitness in a multispecies mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 17234-17239.
Paradis E, Claude J, Strimmer K (2004) APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics*, **20**, 289-290.
Parks JC, Werth CR (1993) A study of spatial features of clones in a population of Bracken Fern, *Pteridium aquilinum* (Dennstaedtiaceae). *American Journal of Botany*, **80**, 537-544.
Paul RG (1977) *Aspects of the biology and taxonomy of British myrmecophilous root aphids*. PhD Thesis, Department of Entomology, Britsh Museum, London.
Peakall R, Ruibal M, Lindenmayer DB (2003) Spatial autocorrelation analysis offers new insights into gene flow in the Australian bush rat, *Rattus fuscipes. Evolution*, **57**, 1182-1195.
Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288-295.
Pen I, Taylor P (2005) Modelling information exchange in worker-queen conflict over sex allocation. *Proceedings of the Royal Society B-Biological Sciences*, **272**, 2403-2408.
Pen I, Weissing F (2000) Towards a unified theory of cooperative breeding: the role of ecology and life history re-examined. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **267**, 2411-2418.
Pepper JW, Smuts BB (2002) A mechanism for the evolution of altruism among nonkin: Positive assortment through environmental feedback. *American Naturalist*, **160**, 205-213.
Perc M, Szolnoki A (2010) Coevolutionary games-A mini review. *Biosystems*, **99**, 109-125.
Pontin AJ (1978) Numbers and distribution of subterranean aphids and their exploitation by the ant *Lasius flavus* (Fabr.). *Ecological Entomology*, **3**, 203-207.
Pontin AJ (1963) Further considerations of competition and the ecology of the ants *Lasius flavus* (F) and *L. niger* (L). *Journal of Animal Ecology*, **32**, 565-574.
Pontin AJ (1961a) Population stabilization and competition between the ants *Lasius flavus* (F) and *L. niger* (L). *Journal of Animal Ecology*, **30**, 47-54.
Pontin AJ (1961b) The prey of *Lasius niger* (L.) and *L. flavus* (F.) (Hym., Formicidae). *The Entomologist's Monthly Magazine*, **97**, 135-137.
Pontin AJ (1959) Some records of predators and parasites adapted to attack aphids attended by ants. *The Entomologist's Monthly Magazine*, **95**, 154-155.

Pontin AJ (1958) A preliminary note on the eating of aphids by ants of the genus *Lasius* (Hym., Formicidae). *The Entomologist's Monthly Magazine*, **94**, 9-11.
Poulsen M (2005) *Conflict and Cooperation in Fungus-growing Ant Mutualisms.* PhD-thesis, University of Copenhagen.
Poulsen M, Boomsma JJ (2005) Mutualistic fungi control crop diversity in fungus-growing ants. *Science*, **307**, 741-744.
Poulsen M, Fernandez-Marin H, Currie CR, Boomsma JJ (2009) Ephemeral windows of opportunity for horizontal transmission of fungal symbionts in leaf-cutting ants. *Evolution*, **63**, 2235-2247.
Q
Queller D (1994) Genetic relatedness in viscous populations. *Evolutionary Ecology*, **8**, 70-73.
Queller DC (1985) Kinship, reciprocity and synergism in the evolution of social behaviour. *Nature*, **318**, 366-367.
Queller DC, Ponte E, Bozzaro S, Strassmann JE (2003) Single-gene greenbeard effects in the social amoeba *Dictyostelium discoideum. Science*, **299**, 105-106.
Queller D (1992) Does population viscosity promote kin selection. *Trends in Ecology & Evolution*, **7**, 322-324.
R
R Development Core Team (2011) R: A language and environment for statistical computing.
Rasmusen E (2006) *Games and information.* Blackwell Publishing Ltd, Oxford.
Raymond M, Rousset F (1995) An exact test for population differentiation. *Evolution*, **49**, 1280-1283.
Réaumur RA (1738) Memoires pour servir a l'histoire de insectes 3:1-312, Amsterdam.
Rispe C, Pierre JS, Simon JC, Gouyon PH (1998) Models of sexual and asexual coexistence in aphids based on constraints. *Journal of Evolutionary Biology*, **11**, 685-701.
Rohlf FJ, Sokal RR (1981) *Statistical tables*, second edn. W.H. Freeman and Company, San Francisco.
Rousset F (2008) GENEPOP ' 007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103-106.
Rozen S, Skaletsky HJ, Krawetz S, Misener S (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* pp. 365-386 Humana Press, Totowa, NJ.
Rütten K, Schulz I, Olek K, Uhl G (2001) Polymorphic microsatellite markers in the spider Pholcus phalangioides isolated from a library enriched for CA repeats. *Molecular Ecology Notes*, **1**, 255-257.
S
Sachs JL, Mueller UG, Wilcox TP, Bull JJ (2004) The evolution of cooperation. *Quarterly Review of Biology*, **79**, 135-160.
Sachs JL, Simms EL (2006) Pathways to mutualism breakdown. *Trends in Ecology & Evolution*, **21**, 585-592.
Sarkar D (2008) *Lattice: multivariate data visualization with R.* Springer, New York.
Schiøtt M, Rogowska-Wrzesinska A, Roepstorff P, Boomsma JJ (2010) Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. *BMC Biology*, **8**, 156.
Seifert B (2007) *Die Ameisen Mittel- und Nordeuropas.* Lutra, Görlitz.
Selten R, Hammerstein P (1984) Gaps in Harley argument on evolutionarily stable learning rules and in the logic of Tit for Tat. *Behavioural and Brain Sciences*, **7**, 115-116.
Severance EG, Karl SA (2006) Contrasting population genetic structures of sympatric, mass-spawning Caribbean corals. *Marine Biology*, **150**, 57-68.
Sherratt T, Roberts G (1998) The evolution of generosity and choosiness in cooperative exchanges. *Journal of Theoretical Biology*, **193**, 167-177.
Simon JC, Baumann S, Sunnucks P, *et al.* (1999) Reproductive mode and population genetic structure of the cereal aphid *Sitobion avenae* studied using phenotypic and microsatellite markers. *Molecular Ecology*, **8**, 531-545.
Simon JC, Carrel E, Hebert PDN, Dedryver CA, Bonhomme J, LeGallic JF (1996) Genetic diversity and mode of reproduction in French populations of the aphid *Rhopalosiphum padi* L. *Heredity*, **76**, 305-313.

Simon JC, Rispe C, Sunnucks P (2002) Ecology and evolution of sex in aphids. *Trends in Ecology & Evolution*, **17**, 34-39.
Smart L (1991) *Asymmetric interactions between ants, aphids and plants*. PhD Thesis, University of Bath.
Smouse PE, Long JC, Sokal RR (1986) Multiple-regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology*, **35**, 627-632.
Smouse PE, Peakall R (1999) Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. *Heredity*, **82**, 561-573.
Stachowicz JJ (2001) Mutualism, facilitation, and the structure of ecological communities. *Bioscience*, **51**, 235-246.
Stadler B, Dixon AFG (2005) Ecology and evolution of aphid-ant interactions. *Annual Review of Ecology Evolution and Systematics*, **36**, 345-372.
Stadler B, Dixon T (2008) *Mutualism: Ants and their insect partners.* Cambridge University Press, Cambridge.
Stamati K, Hollingsworth PM, Russell J (2007) Patterns of clonal diversity in three species of sub-arctic willow (*Salix lanata*, *Salix lapponum* and *Salix herbacea*). *Plant Systematics and Evolution*, **269**, 75-88.
Stenberg P, Lundmark M, Knutelski S, Saura A (2003a) Evolution of clonality and polyploidy in a weevil system. *Molecular Biology and Evolution*, **20**, 1626-1632.
Stenberg P, Lundmark M, Saura A (2003b) MLGsim: a program for detecting clones using a simulation approach. *Molecular Ecology Notes*, **3**, 329-331.
Stoeckel S, Grange J, Fernandez-Manjarres JF, Bilger I, Frascaria-Lacoste N, Mariette S (2006) Heterozygote excess in a self-incompatible and partially clonal forest tree species - *Prunus avium* L. *Molecular Ecology*, **15**, 2109-2118.
Sunnucks P, DeBarro PJ, Lushai G, Maclean N, Hales D (1997) Genetic structure of an aphid studied using microsatellites: Cyclic parthenogenesis, differentiated lineages and host specialization. *Molecular Ecology*, **6**, 1059-1073.

T

Taylor PD (1988) An inclusive fitness model for dispersal of offspring. *Journal of Theoretical Biology*, **130**, 363-378.
Taylor P (1992) Altruism in viscous populations - an inclusive fitness model. *Evolutionary Ecology*, **6**, 352-356.
Teacher AGF, Griffiths DJ (2011) HapStar: automated haplotype network layout and visualization. *Molecular Ecology Resources*, **11**, 151-153.
Tegelaar K, Hagman M, Glinwood R, Pettersson J, Leimar O (2011) Ant-aphid mutualism: the influence of ants on the aphid summer cycle. *Oikos*, **121**, 61-66.
Thompson J, Fernandez C (2006) Temporal dynamics of antagonism and mutualism in a geographically variable plant-insect interaction. *Ecology*, **87**, 103-112.
Tibayrenc M, Kjellberg F, Ayala FJ (1990) A clonal theory of parasitic protozoa: The population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2414-2418.
Trivers RL (1971) Evolution of reciprocal altruism. *Quarterly Review of Biology*, **46**, 35-56.

V

van Boheemen LA (2010) *Ecological stress interacting with genetic diversity and fitness consequences of an eusocial insect*. M.Sc. Thesis, University of Groningen, Groningen.
van Borm S, Buschinger A, Boomsma J, Billen J (2002) Tetraponera ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **269**, 2023-2027.
Van Damme E (1991) *Stability and Perfection of Nash Equilibria*, 2nd edn. Springer-Verlag, Berlin.
Van Doorn G, Hengeveld G, Weissing F (2003) The evolution of social dominance - I: Two-player models. *Behaviour*, **140**, 1305-1332.
Vantaux A, Billen J, Wenseleers T (2011a) Levels of clonal mixing in the black bean aphid *Aphis fabae*, a facultative ant mutualist. *Molecular Ecology*, **20**, 4772-4785.

Vantaux A, Parmentier T, Billen J, Wenseleers T (2012) Do *Lasius niger* ants punish low-quality black bean aphid mutualists? *Animal Behaviour*, **83**, 257-262.
Vantaux A, Van den Ende W, Billen J, Wenseleers T (2011b) Large interclone differences in melezitose secretion in the facultatively ant-tended black bean aphid *Aphis fabae*. *Journal of Insect Physiology*, **57**, 1614-1621.
Veen GF (2011) *Living apart together - Interactions between aboveground grazers, plants and soil biota*. PhD Thesis, University of Groningen, Groningen.
Verbruggen E, El Mouden C, Jansa J, *et al*. (2012) Spatial Structure and Interspecific Cooperation: Theory and an Empirical Test Using the Mycorrhizal Mutualism. *American Naturalist*, **179**, E133-E146.
Villate L, Esmenjaud D, Van Helden M, Stoeckel S, Plantard O (2010) Genetic signature of amphimixis allows for the detection and fine scale localization of sexual reproduction events in a mainly parthenogenetic nematode. *Molecular Ecology*, **19**, 856-873.
Völkl W, Woodring J, Fischer M, Lorenz MW, Hoffmann KH (1999) Ant-aphid mutualisms: the impact of honeydew production and honeydew sugar composition on ant preferences. *Oecologia*, **118**, 483-491.
Vorburger C (2006) Temporal dynamics of genotypic diversity reveal strong clonal selection in the aphid *Myzus persicae*. *Journal of Evolutionary Biology*, **19**, 97-107.

W

WallisDeVries MF, Poschlod P, Willems JH (2002) Challenges for the conservation of calcareous grasslands in northwestern Europe: integrating the requirements of flora and fauna. *Biological Conservation*, **104**, 265-273.
Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, **10**, 506-513.
Way MJ (1963) Mutualism between ants and honeydew-producing Homoptera. *Annual Review of Entomology*, **8**, 307-344.
Weber NA (1972) Fungus-culturing behaviour of ants. *American Zoologist*, **12**, 577-587.
Weir BS, Cockerham CC (1984) Estimating F-Statistics for the analysis of population structure. *Evolution*, **38**, 1358-1370.
Welch D, Meselson M (2000) Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. *Science*, **288**, 1211-1215.
Weng Y, Azhaguvel P, Michels GJ, Rudd JC (2007) Cross-species transferability of microsatellite markers from six aphid (Hemiptera: Aphididae) species and their use for evaluating biotypic diversity in two cereal aphids. *Insect Molecular Biology*, **16**, 613-622.
West SA, Griffin AS, Gardner A (2007) Evolutionary explanations for cooperation. *Current Biology*, **17**, R661-R672.
West SA, Herre EA (1994) The ecology of the new-world fig-parasitizing wasps Idarnes and implications for the evolution of the fig-pollinator mutualism. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **258**, 67-72.
West S, Pen I, Griffin A (2002) Conflict and cooperation - Cooperation and competition between relatives. *Science*, **296**, 72-75.
West-Eberhard M (1975) Evolution of social behaviour by kin selection. *Quarterly Review of Biology*, **50**, 1-33.
Weyl EG, Frederickson ME, Yu DW, Pierce NE (2010) Economic contract theory tests models of mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 15712-15716.
Weyl EG, Frederickson ME, Yu DW, Pierce NE (2011) Reply to Kiers *et al.*: Economic and biological clarity in the theory of mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, E8-E8.
Wolf M, Van Doorn GS, Weissing FJ (2011) On the coevolution of social responsiveness and behavioural consistency. *Proceedings of the Royal Society B-Biological Sciences*, **278**, 440-448.

Woodring J, Wiedemann R, Fischer MK, Hoffmann KH, Volkl W (2004) Honeydew amino acids in relation to sugars and their role in the establishment of ant-attendance hierarchy in eight species of aphids feeding on tansy (*Tanacetum vulgare*). *Physiological Entomology*, **29**, 311-319.

Wulff JL (1985) Clonal organisms and the evolution of mutualism. In: *Population biology and evolution of clonal organisms* (eds. Jackson JBC, Buss LW, Cook RE) pp. 437-466 Yale University Press, New Haven, CT.

X

Xhaard C, Fabre B, Andrieux A, et al (2011) The genetic structure of the plant pathogenic fungus *Melampsora larici-populina* on its wild host is extensively impacted by host domestication. *Molecular Ecology*, **20**, 2739-2755.

Y

Yamamura N (1993) Vertical transmission and evolution of mutualism from parasitism. *Theoretical Population Biology*, **44**, 95-109.

Yamamura N, Higashi M, Behera N, Wakano JY (2004) Evolution of mutualism through spatial effects. *Journal of Theoretical Biology*, **226**, 421-428.

Yao I (2010) Contrasting patterns of genetic structure and dispersal ability in ant-attended and non-attended *Tuberculatus* aphids. *Biology letters*, **6**, 282-286.

Yao I, Akimoto S (2009) Seasonal changes in the genetic structure of an aphid-ant mutualism as revealed using microsatellite analysis of the aphid *Tuberculatus quercicola* and the ant *Formica yessensis*. *Journal of Insect Science*, **9**, 1-18.

Yao I, Akimoto S (2001) Ant attendance changes the sugar composition of the honeydew of the drepanosiphid aphid *Tuberculatus quercicola*. *Oecologia*, **128**, 36-43.

Yonezawa K, Ishii T, Nagamine T (2004) The effective size of mixed sexually and asexually reproducing populations. *Genetics*, **166**, 1529-1539.

Young AG, Hill JH, Murray BG, Peakall R (2002) Breeding system, genetic diversity and clonal structure in the sub-alpine forb *Rutidosis leiolepis* F. Muell. (Asteraceae). *Biological Conservation*, **106**, 71-78.

Z

Zhang C, Zhang J, Weissing FJ, Perc M, Xie G, Wang L (2012) Diferent reactions to adverse neighbourshoods in games of cooperation. *Plos One*, **7**, e35183.

Zwölfer H (1958) Zur systematik, biologie, and ökologie unterirdisch lebender Aphiden (Homoptera, Aphidoidea). *Zeitschrift für angewandte Entomologie*, **40**, 182-221.

Samenvatting

MUTUALISME

Mutualisme, een interactie tussen organismen die tot verschillende soorten behoren en waarvan beide 'partners' profijt hebben, komt veel voor in de natuur. We kunnen zelfs stellen dat mutualismen het leven op aarde zoals we dat kennen over miljoenen jaren hebben beïnvloed en ook nu nog steeds beïnvloeden. Zo wordt bijvoorbeeld de energievoorziening in de cellen van de meeste organismen verzorgd door mitochondriën die lang geleden nog onafhankelijke, mutualistische bacteriën waren. Mutualismen in allerlei vormen en maten zijn ook belangrijk voor de stabiliteit van ecosystemen in onze veranderende wereld; denk aan planten en hun bestuivende insecten en de verspreiding van zaden door vogels en knaagdieren. Andere mutualismen die iedere dag weer van invloed zijn op ons leven zijn de interacties tussen ons en ons vee en onze gewassen, maar ook bijvoorbeeld onze 'samenwerking' met de bacteriën in onze darmen: zij helpen de vertering van ons voedsel in ruil voor veilige 'woonruimte' en continue aanvoer van hun eigen nutriënten.

Ondanks het overduidelijke belang van mutualisme voor het leven op onze planeet is dit proces historisch onderbelicht gebleven in de evolutiebiologie; traditioneel kregen processen als competitie en gastheer-parasiet-interacties meer aandacht. Pas sinds de laatste twee decennia beseffen evolutiebiologen dat het ontstaan van mutualismen en hun stabiliteit over evolutionaire tijd niet vanzelfsprekend zijn en tot op heden onbegrepen zijn gebleven.

Het doel van dit proefschrift was om meer inzicht te krijgen in de evolutie en ecologie van mutualismen. Om deze inzichten te krijgen maakte ik gebruik van twee complementaire invalshoeken. Allereerst deed ik **empirisch**, (moleculair) **ecologisch onderzoek** aan één specifiek mutualisme, dat tussen de Gele weidemier en de wortelbladluizen in haar nest. Ten tweede combineerde ik dit met **theoretisch** onderzoek in de vorm van simulatiemodellen die de evolutie van samenwerking binnen en tussen soorten nabootsen.

DE PUZZEL DIE DE EVOLUTIE VAN SAMENWERKING HEET

De evolutie van samenwerking is één van de grootste vraagstukken binnen de evolutietheorie. Natuurlijke selectie is immers gebaseerd op competitie om voortplanting en overleving. Hoe hebben deze concurrerende organismen dan toch het vermogen ontwikkeld elkaar te helpen, terwijl ze vaak meer voordeel zouden kunnen behalen wanneer ze alleen van anderen zouden profiteren zonder zelf te investeren?

Samenwerking binnen soorten

Dit probleem is zeer uitgebreid onderzocht voor het geval van samenwerking tussen soortgenoten ('binnen soorten'). De belangrijkste mechanismen die tot de evolutie van samenwerking kunnen leiden kunnen worden onderverdeeld in twee categorieën:

(1) De eerste categorie bestaat uit mechanismen die ervoor zorgen dat potentiële samenwerkingspartners niet willekeurig bij elkaar komen. Een voorbeeld van een dergelijk mechanisme is het zogeheten *kin selection*, waarbij samenwerking tussen familieleden

als bijkomend voordeel heeft dat gemeenschappelijke genen worden doorgegeven. Een ander voorbeeld is samenwerking die kan ontstaan wanneer individuele organismen, die de neiging hebben tot helpen, bij elkaar in de buurt blijven, bijvoorbeeld omdat ze zich zelden verspreiden.

(2) De tweede categorie bevat mechanismen die de investering in samenwerking af laten hangen van de acties van anderen of van omgevingskenmerken. Een voorbeeld hiervan is *Tit-for-Tat*:'voor wat, hoort wat'.

Samenwerking tussen soorten

Kin selection bleek één van de beste verklaringen te zijn voor samenwerking tussen soortgenoten. Voor mutualisme, de samenwerking tussen organismen van verschillende soorten ('tussen soorten'), kan *kin selection* echter geen rol spelen: verschillende soorten delen hun genen per definitie niet. Het bestaan van mutualisme is daarom tot nu toe onverklaard gebleven en wordt gezien als één van de grote onopgeloste vraagstukken binnen de evolutiebiologie. Dit vraagstuk is tweeledig: (1) Ten eerste is het onduidelijk hoe mutualismen kunnen *ontstaan*. (2) Ten tweede is het onduidelijk hoe mutualismen als zodanig *in stand blijven*. Dit laatste kan namelijk problematisch zijn omdat natuurlijke selectie altijd op beide partners apart van toepassing zal zijn en niet op de interactie als geheel. Dat betekent dat partners door natuurlijke selectie zullen worden geselecteerd om zoveel mogelijk van de interactie te profiteren en er zelf zo weinig mogelijk in te investeren. Met andere woorden: theoretisch zouden mutualisten door natuurlijke selectie moeten worden gedreven tot het parasiteren van hun partner. Toch wijst het wijdverbreid voorkomen van mutualisme erop dat een mutualistische interactie niet zomaar een parasitaire interactie wordt. Welke mechanismen zorgen ervoor dat dit niet gebeurt? En welke mechanismen zorgen er eigenlijk voor dat die mutualismen überhaupt vanuit het niets kunnen ontstaan? Hieronder zal ik de voornaamste theoretische ideeën over het ontstaan en in stand blijven van mutualismen behandelen.

(1) Dit eerste idee betreft voornamelijk het ontstaan van mutualismen. Veel mutualismen komen waarschijnlijk voort uit *bijproduct-interacties*. Dit zijn interacties waarbij er bij een handeling van de ene soort een bijproduct ontstaat dat van waarde is voor een andere soort. Belangrijke voorbeelden hiervan zijn honingdauwmutualismen. Hierbij scheiden insecten die leven op plantensappen (bijvoorbeeld bladluizen) een suikerachtig goedje uit (honingdauw) als uitwerpselen. Deze honingdauw wordt dan weer opgegeten door bijvoorbeeld mieren, die in ruil daarvoor goed voor de bladluizen zorgen. In zulke gevallen is de verhouding tussen kosten en baten van de interactie voor de producent van het bijproduct laag: de luizen maken de honingdauw vrijwel gratis en krijgen er waardevolle bescherming voor terug.

(2) Het tweede idee heeft voornamelijk betrekking op het in stand blijven van mutualismen. Dit betreft twee mechanismen die te maken hebben met *wederkerigheid* van gedrag tussen de partners. Deze mechanismen zijn de *betrouwbaarheid* van een partner over tijd en *partnerkeuze en sancties* tegen partners die niet samenwerken. Met betrouwbaarheid wordt bedoeld dat de ene partner er voldoende op kan rekenen dat de andere partner bij de volgende gelegenheid tot interactie ook weer beschikbaar is. Als deze betrouwbaarheid hoog is, kunnen partners op elkaar rekenen en is er een

goede basis voor mutualistische samenwerking in de toekomst. In de natuur komt een dergelijk hoge betrouwbaarheid voor wanneer twee samenwerkende soorten zich na voorplanting samen verspreiden, zoals bijvoorbeeld bacteriën die in het lichaam van insecten leven en zich tegelijkertijd voortplanten met deze insecten. Partnerkeuze en sancties zorgen ervoor dat partners actief kunnen kiezen samen te werken met 'goede' partners en 'slechte' partners kunnen vermijden. Ook op deze manier ontstaat er een goede basis voor samenwerking. Een voorbeeld van partnerkeuze en sancties in de natuur kan gevonden worden in de interactie tussen planten en bacteriën die stikstof voor hen fixeren in ruil voor voedingsstoffen: deze planten geven alleen voedingsstoffen aan de bacteriën die het best presteren.

HET 'KWEEKMUTUALISME' TUSSEN DE GELE WEIDEMIER EN WORTELBLADLUIZEN

Kweekmutualismen

Het empirische deel van dit proefschrift staat in het teken van één specifieke mutualistische interactie: die tussen de Gele weidemier *Lasius flavus* en meerdere soorten wortelbladluizen die door deze mier in haar nest worden gehouden. Deze interactie vertegenwoordigt een bepaalde groep van mutualismen: de zogeheten 'kweekmutualismen'. In dergelijke interacties bevordert de ene soort, de 'kweker', de groei van de andere soort, de 'symbiont', van wie de kweker afhankelijk is voor de voedselvoorziening. Kweekmutualismen komen in allerlei vormen en maten voor: Er zijn mieren en termieten die schimmels kweken in hun nest, in de zee houden bepaalde vissen keurige algentuintjes bij en er zijn zelfs ééncellige amoeben bekend die bacteriën kweken.

De Gele weidemier en haar wortelbladluizen

Het systeem dat ik hier bestudeerde is een voorbeeld van veehouderij in de natuur: de Gele weidemier bouwt in haar ondergrondse nesten kleine kamertjes die een beschermde omgeving vormen waar de wortelbladluizen rustig van plantenwortels kunnen drinken. De Gele weidemier is afhankelijk van de luizen voor zowel suiker als eiwit: de mieren 'melken' de luizen voor het suikerachtige goedje honingdauw en 'slachten' hun vee ook af en toe voor hun stikstofvoorziening. Andersom zijn ook de luizen afhankelijk geworden van deze interactie: de lichaamsbouw van de luizen is volledig aangepast aan het leven bij de mieren, waardoor ze niet meer onafhankelijk kunnen overleven. Er kunnen meer dan dertien verschillende soorten luizen in de mierennesten voorkomen. De Gele weidemieren en hun wortelbladluizen zijn op meerdere plekken in noordwest Europa te vinden. Slechts onder zeer specifieke ecologische omstandigheden maken ze hun grote karakteristieke mierenbulten. De kwelder van Schiermonnikoog is een dergelijke locatie en het meeste veldwerk voor dit proefschrift heeft dan ook daar plaatsgevonden.

Mogelijke conflicten tussen de partners

De insteek van dit gedetailleerde onderzoek naar de evolutionaire ecologie van de Gele weidemier-interactie was om de inzichten over dit kweekmutualisme te vergelijken met

gegevens van andere dergelijke interacties om op deze manier nieuwe algemene inzichten over de ecologie en evolutie van kweekmutualismen te krijgen. De achtergrondgedachte hierbij is dat in dergelijke interacties beide partners er altijd een eigen agenda op zullen nahouden. Voor de kweker is het bijvoorbeeld van belang dat hij erop kan rekenen dat de symbiont zoveel mogelijk in de buurt blijft en zoveel mogelijk energie investeert in het produceren van voedsel, terwijl de symbiont misschien ook energie wil investeren in onafhankelijke verspreiding of seksuele voortplanting (in plaats van bijvoorbeeld klonale voortplanting). Deze eigen agenda's zouden mogelijk tot evolutionaire conflicten kunnen leiden tussen de beide parters. In het onderzoek van dit proefschrift stonden drie mogelijke conflicten tussen kweker en symbiont centraal: de potentiële conflicten over (1) de voortplantingswijze van de symbiont (sexueel of asexueel/klonaal), (2) de mate van verspreiding van de symbiont (veel of weinig) en (3) de (genetische) diversiteit van de symbiontenpopulatie (mengcultuur of monocultuur).

RESULTATEN VAN DE EMPIRISCHE STUDIES

Hoofdstuk 2 richtte zich op de wijze van voortplanting en mate van verspreiding van de vier meest voorkomende wortelbladluissoorten in de nesten van *Lasius flavus*. In deze studie verzamelde ik wortelbladluizen uit mierenbulten op de kwelder van Schiermonnikoog. Door middel van een populatiegenetische analyse, die ik verder toelicht in **hoofdstuk 3**, toonde ik aan dat alle vier de luizensoorten zich voornamelijk (en mogelijk alleen maar) klonaal voortplanten in deze eilandpopulatie. Luizenmoeders produceren dus enkel dochters die exacte kopieën zijn van zichzelf. Ook toonde ik aan dat de luizen zich maar zelden verspreiden vanuit de mierennesten: over het algemeen komen specifieke klonen slechts zeer lokaal voor. Deze bevinding werd verder ondersteund door de waarneming dat gevleugelde luizen zeer zeldzaam zijn. Interessant genoeg is deze combinatie van klonale voortplanting en weinig verspreiding ook veelvoorkomend in andere kweekmutualismen. Ook de schimmel van schimmelkwekende mieren plant zich enkel klonaal voort en verspreiding die onafhankelijk van de verspreiding van mieren is, komt maar zelden voor.

In **hoofdstuk 4** bracht ik de diversiteit aan luizensoorten en luizenklonen in de mierenbulten verder in kaart. Deze studie laat zien dat in de meerderheid van de mierenbulten slechts één luizensoort voorkomt en dat er binnen die soort vaak maar één enkele kloon per mierennest voorkomt. Wanneer er meerdere soorten en/of klonen in een nest voorkomen, leven deze vaak gescheiden in aparte luizenkamers. Op bult-niveau is er dus soms sprake van mengculturen, maar op luizenkamer-niveau komen er bijna uitsluitend monoculturen voor. Deze vinding van monoculturen is in lijn met andere kweekmutualismen, zoals bijvoorbeeld de vissen met hun algentuinen die vaak slechts één soort alg kweken. Hoofdstuk 4 toont verder aan dat het aannemelijk is dat de mieren voornamelijk jonge luizen opeten voor hun eiwitvoorziening en een beperkter aantal van luizen (ongeveer 70) per bult bewaren voor de honingdauwvoorziening. De pilot-studie van **Box A** laat zien dat mieren in keuzeexperimenten geen voorkeur laten zien voor luizen van verschillende soort of herkomst. Deze resultaten suggereren dat er wellicht geen actieve partnerkeuze door mieren aan deze lage diversiteit ten grondslag ligt. In plaats daarvan kan deze lage diver-

siteit verklaard worden uit de klonale voortplanting van de luizen en beperkte verspreiding die daarop volgt.

Tot slot wordt de ontwikkeling van de genetische markers die gebruikt werden voor de studies van hoofdstukken 2 en 4 verder toegelicht in **hoofdstuk 5**. Tevens wordt in **Box B** de ontwikkeling van de software die gebruikt is voor deze genetische analyse toegelicht.

RESULTATEN VAN DE THEORETISCHE MODELLEN

In **hoofdstukken 6** en **7** bestudeerde ik de evolutie van samenwerking binnen soorten en tussen soorten met behulp van computersimulaties. In **hoofdstuk 6** ontwierp ik een model voor samenwerking binnen één soort, waarbij ik als voorbeeld bacteriën in gedachten hield. In het model vormen deze bacteriën één enkele populatie, die is onderverdeeld in subpopulaties. De bacteriën kunnen een stofje uitscheiden dat voor hen kostbaar is om te maken, maar waar zijzelf en hun soortgenoten van kunnen groeien. De totale hoeveelheid van het stofje wordt binnen de subpopulatie gedeeld. In het model liet ik de neiging tot investeren in het stofje samen evolueren met de neiging om de thuisbasis te verruilen voor een andere subpopulatie op basis van de hoeveelheid van het stofje die beschikbaar was. Hierbij verwachtte ik dat individuen beter zouden samenwerken (lees: meer van het stofje zouden maken) als iedereen de neiging had weg te gaan uit 'slechte' subpopulaties. Het tegendeel bleek waar: in dit model bleek samenwerking juist te ontstaan wanneer de bacteriën 'goede' subpopulaties verruilden voor 'slechte'. Deze uitkomst is demografisch goed te verklaren: bacteriën die zijn opgegroeid in een goede subpopulatie verkeren in een dusdanig goede gezondheid dat ze de bacteriën in een slechte subpopulatie gemakkelijk kunnen wegconcurreren als ze daarheen verhuizen. Kortom, zulke modellen die gebruik maken van de evolutie van gedrag dat afhankelijk is van de acties van anderen kunnen vaak tot verrassende nieuwe inzichten leiden.

Dit gold ook voor de resultaten van het model uit **hoofdstuk 7**. De basis van dit model was dezelfde als die van hoofdstuk 6, maar in deze simulaties modelleerde ik populaties van twee verschillende soorten bacteriën die met elkaar samenwerken, een voorbeeld van mutualisme dus. Beide soorten scheidden nu een stofje uit dat als voedsel diende voor de andere soort, maar niet voor henzelf. Iedere subpopulatie bestond uit bacteriën van beide soorten en de keuze om te blijven of te vertrekken was gebaseerd op hoeveel de bacteriën van de andere soort van het stofje produceerden. Ook konden bacteriën op basis hiervan kiezen naar welke nieuwe subpopulatie ze wilden gaan. Ook in dit model bleek samenwerking te evolueren wanneer bacteriën besloten weg te gaan uit goede subpopulaties. Dit model bevestigde ook het belang van relatief lage kosten ten opzichte van baten: wanneer de kosten in verhouding hoog werden, evolueerde samenwerking niet meer. Interessant genoeg bleken de mutualistische interacties die evolueerden vaak asymmetrisch te zijn: de ene bacteriesoort investeerde vaak meer dan de ander. Deze asymmetrie is een bekend fenomeen uit de natuur en is ook terug te zien in de verschillen tussen kwekers en symbionten in kweekmutualismen.

CONCLUSIE

De empirische resultaten geven aan dat het voor de evolutie van mutualisme van belang is dat (generaties van) partners gedurende langere tijdsspannes op elkaar kunnen rekenen (*betrouwbaarheid*). In de interactie van de Gele weidemier met haar wortelbladluizen komt dit sterk naar voren in de vorm van weinig verspreiding en weinig genetische diversiteit: de mieren kunnen dus min of meer rekenen op constante aanwezigheid van min of meer dezelfde luizen. Anderzijds geven aanpassingen in lichaamsbouw van de luizen en het opgeven van sexuele reproductie aan dat ook de luizen in de loop van de tijd zijn gaan 'rekenen' op de aanwezigheid van de mieren.

De theoretische resultaten wijzen er echter op dat deze dynamiek van op elkaar kunnen rekenen alleen van toepassing is onder specifieke populatiekenmerken: als partners zich pas verspreiden na de vruchten te hebben geplukt van de samenwerking kan het evolutionair ook voordelig zijn juist bij goede partners weg te gaan.

Zowel de empirische als de theoretische resultaten wijzen erop dat het aannemelijk is dat relatief lage kosten ten op zichte van de baten ten grondslag liggen aan de evolutie van mutualismen (zogeheten *bijproduct-mutualismen*). Het is aannemelijk dat het honingdauw-mutualisme tussen de mieren en de luizen ooit ontstaan is vanuit mieren die de uitwerpselen van luizen als voedingsbron ging benutten. Vanuit deze – voor de luizen- gratis interactie kon het systeem zich verder ontwikkelen tot de huidige vorm van onderlinge afhankelijkheid van de mieren en de luizen. Deze bevinding wordt onderschreven door de resulaten van de simulatiemodellen: samenwerking in de natuur kan enkel van de grond komen als de voordelen aanzienlijk groter zijn dan de gevraagde investering.

Curriculum Vitae

Aniek Ivens (1983) groeide op in het Limburgse Sittard. Na afronding van het gymnasium aan Trevianum Sittard (2001, summa cum laude) verruilde ze Sittard voor Wageningen voor haar studie Biologie. Tijdens deze Bachelor of Science specialiseerde zij zich in (moleculaire) ecologie en ontdekte evolutie als haar favoriete niche binnen de biologie. In 2004 studeerde zij (cum laude) af. Vervolgens verhuisde ze wederom noordwaarts naar Groningen waar zij deel uitmaakte van het Topmaster-programma Evolutionary Biology. Deze studie richtte zich op het grensvlak van de evolutie en ecologie. In 2006 studeerde zij succesvol (cum laude) af op zowel een onderzoek naar soortsherkenning in sluipwespen als een onderzoek naar het mutualisme tussen bladsnijdersmieren en de door hen gekweekte schimmel. Het verblijf in Kopenhagen voor dit tweede afstudeeronderzoek vormde de basis voor het promotieonderzoek gepresenteerd in dit proefschrift. Momenteel is Aniek werkzaam in Amsterdam, waar zij als deelneemster aan de Nationale DenkTank 2012 bijdraagt aan het verduurzamen van de voedselketen in Nederland.

Aniek Ivens (1983) grew up in Sittard, The Netherlands. After graduating from grammarschool Trevianum Sittard (graduated summa cum laude, 2001), she moved to Wageningen to study Biology at Wageningen University and Research Centre. During her B.Sc. degree she specialized in (molecular) ecology and discovered Evolution as her favourite niche within Biology. She graduated cum laude in 2004. After this, she moved Northwards once again where she enrolled in the Topmaster's programme Evolutionary Biology. This M.Sc. programme focussed on the interplay between ecology and evolution. She graduated cum laude in 2006 with two M.Sc. projects: the first dealing with species recognition in parasitoid wasps and the second dealing with the mutualism between leaf-cutter ants and the fungus they grow in their nests. The stay in Copenhagen for this second project became the foundation of the PhD-research presented in this thesis. Currently, Aniek works in Amsterdam where she, as a participant of this year's National Think Tank, hopes to contribute to making the food chain in the Netherlands more sustainable.

Author affiliations and contact information

Aniek B.F. Ivens
- Theoretical Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
- Centre for Social Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen, Denmark.

E-mail: A.B.F.Ivens@rug.nl

Daniel J.C. Kronauer
Laboratory of Insect Social Evolution, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA.
E-mail: dkronauer@mail.rockefeller.edu

Franz J. Weissing
Theoretical Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
E-mail: F.J.Weissing@rug.nl

Ido Pen
Theoretical Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
E-mail: I.R.Pen@rug.nl

Jacobus J. Boomsma
Centre for Social Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen, Denmark
E-mail: jjboomsma@bi.ku.dk

Joke Bakker
Theoretical Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
E-mail: joke.bakker@rug.nl

Marten van de Sanden
mart.vd.sanden@gmail.com